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# Studies on the expression and function of *Pseudomonas aeruginosa* pili

by

Barbara-Ann Dorothea Conway ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of Doctor of Philosophy

Department of Microbiology

Edmonton, Alberta
Spring 1996

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Department of Biological Sciences
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Edmonton

Department of Biological Sciences
Faculty of Science

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# Abstract

Studies on the expression and function of *Pseudomonas aeruginosa* pili required an evaluation of pili-mediated adherence to human epithelial cells. Bioluminescence was investigated as a method of bacterial enumeration to support the development of an epithelial cell attachment assay for *P. aeruginosa*. Further, the function of the PilG gene product in pilus assembly was investigated.

Bioluminescence from *P. aeruginosa* expressing cloned *Vibrio fischeri lux* genes was investigated as a method for enumerating bacteria in liquid culture. A linear relationship existed between the light output from the bacteria and the concentration of bacteria in a liquid suspension. Light output was found to be dependent on temperature with the maximum output occurring at room temperature and continuously decreasing up to 30°C and 37°C. This also resulted in unstable light output when the bacteria were subject to a temperature change, precluding the use of this light system for bacterial enumeration in an adherence assay where a 37°C temperature is required.

An A549 human lung pneumocyte adherence assay was developed to characterize the attachment of *P. aeruginosa* to an epithelial cell monolayer and to allow comparisons between different bacterial strains or adherence mutants. The A549 human lung pneumocytes provided a uniform, living attachment surface and a plate count method for bacterial enumeration ensured that only viable bacteria would be counted. A mutant strain of *P. aeruginosa* PAO1 that expressed pilin lacking the C-terminal disulfide loop adherence domain, was able to assemble pili that were morphologically indistinguishable from wild-type, but was not able to adhere to A549 cells at a wild-type level. This mutant was also far less virulent than the wild-type in a mouse model, providing direct evidence that the C-

terminal disulfide loop is important for the adherence properties of pili and the virulence of *P. aeruginosa*, but it is not required for pilus assembly.

The study of mutants in pilus assembly led to the characterization of an insertion mutant and a point mutant in the *pilG* gene of *P. aeruginosa* PAO1. The insertion mutant, unable to synthesize PilG, was non-piliated, incapable of twitching motility, and broadly resistant to pilus-specific bacteriophages. It was also less competent in adherence to A549 cells than wild-type. The *pilG* point mutant was incapable of twitching motility, but retained sensitivity to some pilus-specific bacteriophages and was occasionally observed to have an intact pilus. The adherence of the point mutant to A549 cells and its virulence in a mouse model was essentially the same as wild-type. PilG shares significant amino acid sequence homology with the enteric CheY chemotaxis response regulator and seems to function as a response regulator in pilus assembly in a manner analogous to the function of CheY in controlling the direction of flagellar rotation. If PilG functions in the same way as CheY, the point mutant's Lys-90 to Glu amino acid change may destabilize a phosphorylation site required for activation of the PilG protein.

These studies have provided more information about the structure and function of *P. aeruginosa* type IV pili and have provided support for the involvement of a chemotaxis-like signal transduction mechanism in the assembly of pili.

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# List of Abbreviations

| | |
|---|---|
| A | Amperes |
| Å | Angstrom |
| $A_{260}$ | Absorbance at 260 nanometers |
| ADP (ATP) | Adenosine diphosphate (-triphosphate) |
| AI | Autoinducer |
| Amino acids | Standard abbreviations and 1-letter abbreviations used |
| AP | Alkaline phosphatase |
| Ap | Ampicillin |
| asialo-$G_{M1}$ | Gangliotetraosylceramide |
| asialo-$G_{M2}$ | Gangliotriaosylceramide |
| | |
| BCA | Bicinchoninic acid |
| BCIP | 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidinium salt |
| BEC | Buccal epithelial cell |
| BSA | Bovine serum albumin |
| | |
| cAMP | Cyclic adenosine monophosphate |
| Cer | Ceramide |
| CF | Cystic fibrosis |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CFU | Colony forming units |
| Cm | Chloramphenicol |
| CMF-PBS | Calcium, magnesium-free phosphate-buffered saline |
| | |
| Da | Daltons |
| DIG | Digoxygenin |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| -A | Adenine |
| -C | Cytosine |
| -G | Guanine |
| -T | Thymine |
| ds | Double stranded |
| DSL | Disulfide loop |
| dUTP | Deoxyuridine-5'-triphosphate |
| | |
| EDTA | Ethylenediaminetetraacetic acid |
| EI | Ending inoculum |
| | |
| FC-PBS | PBS, pH7.2 + 1% (v/v) fetal bovine serum + 1% (v/v) $CaCl_2$ |
| | |
| g | Gram |
| Gal | Galactose |
| Glc | Glucose |
| | |
| h | Hour |
| HBSS | Hank's balanced salt solution |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| | |
| IgG | Immunoglobulin G |
| IM | Inner membrane |

| | |
|---|---|
| IPTG | Isopropyl β-D-thiogalactopyranoside |
| k | Kilo- (unit prefix = $10^3$) |
| $K_A$ | Equilibrium association constant |
| $k_a$ | Rate association constant |
| $K_D$ | Equilibrium dissociation constant |
| $k_d$ | Rate dissociation constant |
| kb | Kilobase |
| Km | Kanamycin |
| L | Ligand |
| l | Litre |
| λ | Wavelength |
| LB | Luria-Bertani medium |
| LBA | Luria-Bertani agar |
| $LD_{50}$ | 50% lethal dose |
| Lumi Phos | Lumi Phos 530 |
| M | Molar |
| m | Metre; also milli- (unit prefix = $10^{-3}$) |
| μ | Micro- (unit prefix = $10^{-6}$) |
| MCP | Methyl-accepting chemotaxis protein |
| Me | Methyl |
| mol | Mole |
| min | Minute |
| mRNA | Messenger ribonucleic acid |
| MSD | Membrane-spanning domain |
| n | Nano- (unit prefix = $10^{-9}$) |
| NAc | N-acetyl |
| NBD | Nucleotide-binding domain |
| NBT | Nitro-blue tetrazolium |
| OM | Outer membrane |
| p | Pico- (unit prefix = $10^{-12}$) |
| $P_i$, $PO_4$ | Inorganic phosphate |
| PAG | Polyacrylamide gel |
| PAGE | Polyacrylamide gel electrophoresis |
| PAK | *Pseudomonas aeruginosa* strain K |
| PAO1 | *Pseudomonas aeruginosa* strain O1 |
| PAO4141 | *Pseudomonas aeruginosa* strain O4141 |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| phage | Bacteriophage |
| R | Receptor |
| r | Resistance |
| $r^2$ | Correlation coefficient |
| $R_T$ | Total receptors |
| RD | Regulatory domain |

| | |
|---|---|
| RFLP | Restriction fragment length polymorphism |
| rpm | Revolutions per minute |
| RR | Response regulator |
| | |
| s, sec | Second |
| SD | Shine-Dalgarno sequence |
| SDS | Sodium dodecylsulfate |
| Sp | Spectinomycin |
| SSC | 150 mM NaCl, 15 mM $Na_3$ citrate |
| SSPE | 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA pH7.7 |
| STA | Silicotungstic acid |
| | |
| $t_{1/2}$ | Half-life |
| $T_{calc}$ | Calculated T-value (statistical T-test) |
| $T_m$ | Melting temperature |
| TAE | Tris-acetate-EDTA |
| TBS | Tris-buffered saline |
| TC | Trypsin control |
| Tc | Tetracycline |
| TE | Tris-EDTA |
| TEM | Transmission electron microscopy |
| Tris | Tris(hydroxymethyl)aminomethane |
| TSB | Tryptic soy broth |
| TTBS | Tween 20-Tris buffered saline |
| | |
| U | Units |
| UV | Ultraviolet |
| | |
| V | Volts |
| v/v | Volume/volume |
| | |
| w/v | Weight/volume |
| | |
| X-Gal | 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside |

# Chapter 1

# Introduction

# A. Introduction

## I. Introduction.

*Pseudomonas aeruginosa* is a Gram negative aerobic organism that can be isolated from soil and water and it is occasionally pathogenic for plants (10). More recently it has become recognized as an important opportunistic human pathogen. In 1983, Bodey *et al* wrote that *P. aeruginosa* had emerged as an important pathogen over the previous two decades since it caused between 10% and 20% of infections in most hospitals (11). It has become an important pathogen among individuals whose host defense mechanisms have been compromised, especially cancer and burn patients, patients who have been hospitalized for an extended period and, perhaps most notably, patients with cystic fibrosis (11, 40). *P. aeruginosa* has also become the most common microorganism implicated in contact lens-associated microbial keratitis (21). An innate resistance to many antibiotics and disinfectants and an ability to produce a number of cell-associated and extracellular virulence factors allows this organism to cause a wide range of infections in different environments within a suitable host (11, 40, 61).

Pili (singular, pilus) are one of the cell-associated virulence factors produced by *P. aeruginosa* and these long, thin, filamentous protein structures are responsible for the attachment of the bacteria to host mucosal surfaces (128). This attachment during colonization is the important first specific interaction between a pathogen and its host (32). In addition to their role in adherence, *P. aeruginosa* pili are also known to function in other processes such as twitching motility (19, 43) and bacteriophage adsorption (20). While much effort has been made to understand pilus architecture and the mechanism of pilus biogenesis in *P. aeruginosa*, considerable work remains to elucidate the complete structure-function relationship of the domains of the pilin subunit to pilus function, and to understand the interplay of gene products required for pilus assembly.

## II. Classification and Pilin Structure.

Bacterial pili are thin, non-flagellar protein filaments found on the surfaces of a number of different types of bacteria, functioning mainly in DNA transfer (eg. F pili of *Escherichia coli*) and adherence (84). Noting that there was not a general agreement on a specific classification scheme for pili, Paranchych and Frost (1988) discussed three major groups of pili: conjugative, adhesive, and NMePhe ( later, type IV) pili (84). These groups take into account morphology, function, and biochemical properties, three criteria that lend themselves to pili classification. These pili are constructed of a protein termed pilin and, in some cases, accessory proteins to complete the pilus adhesin, depending on the organism. Investigators have also identified families of pili based on the specificity of host-receptor recognition, seroreactivity of antibodies against the pilin proteins making up the pili, and by comparing the deduced amino acid sequences of cloned pilin structural genes.

The pili of *P. aeruginosa* are of the type IV family of pili (82). These function mainly in adhesion and have not been implicated in conjugal DNA transfer (116). Pili from many different organisms, including *Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Dichelobacter* (formerly *Bacteroides*) *nodosus* and *Vibrio cholera*, in addition to *P. aeruginosa*, belong to this family and they are grouped together on the basis of similarities in amino acid sequence of the pilin protein (88, 116). The region of highest homology occurs at the N-terminus of the pilin protein, encompassing approximately the first third of the 144 amino acid protein (*P. aeruginosa* PAK) (90, 108). In addition, pilin is synthesized as a prepilin precursor which also includes a short (6 residue), positively charged leader peptide that is cleaved to produce the mature pilin protein. The mature form of pilin is characterized by a number of features including an invariant N-methylated amino acid (phenylalanine or methionine) as the first residue (36). Another characteristic of mature pilin is a disulfide loop formed by two oxidized cysteine residues near the C-terminus of the protein with the loop being formed by the 12 to 30 or 40 amino acids (depending on the organism and strain) between the cysteine residues. The disulfide loop has been implicated in bacterial attachment and bacteriophage adsorption (56, 85, 87). A third characteristic of at least some type IV pilins is glycosylation of the pilin protein. Recently, the atomic resolution structure of a 17.4 kDa pilin protein from *Neisseria gonorrhoeae* strain MS11 was solved and this X-ray crystal structure shows the presence of a covalently bound, *O*-linked, *N*-acetyl glucosamine-$\alpha$1,3-galactose at Ser 63 of the protein (88). Castric (1995) also demonstrated that pilin from *P. aeruginosa* strain 1244 was glycosylated, unlike pilin from strains PAO and PAK which have been shown to contain no sugar residues (22, 37, 86). The purpose of glycosylation may be simply to provide antigenic variation with respect to its presence or absence and also the site of glycosylation. The absence of glycosylation seems to have no effect on either the formation of pili or their function (22, 88).

The X-ray crystal structure of the pilin protein from *N. gonorrhoeae* also shows that this type IV pilin has an elongated, ladle-shaped structure with the dimensions 85 x 34 x 26 Å (88). It shares a close relationship with the pilin of *P. aeruginosa* as demonstrated by the ability of *P. aeruginosa* to assemble pili from the *N. gonorrhoeae* pilin subunits (51).

Some features of the *N. gonorrhoeae* pilin that are likely shared with *P. aeruginosa* pilin include a long, hydrophobic $\alpha_1$-helix (residues 2-54 of *N. gonorrhoeae* pilin) that makes up the "ladle handle", and a region of homology in the $\alpha_4$-$\beta_3$ turn of part of the "ladle head" portion of the protein (see Fig. 1.1). These regions of the two proteins share greater than 67% similarity in amino acid sequence (116), indicating a good probability of common function for these conserved areas of the proteins. Fig. 1.1 also shows apparent surface domains of the pilin protein from *P. aeruginosa* PAK identified using immunological studies with native pilin peptides (87, 109), synthetic peptides (66), secondary-structure predictions (83, 109), and mutations conferring resistance to bacteriophages (85). This information supports the idea that the long hydrophobic $\alpha_1$-helix structure is not exposed in native pili and that the majority of the surface exposed regions of pilin occur in the "ladle head" structure since the putative surface exposed areas are either in the head regions or at the C-terminal end of the $\alpha_1$-helix which is close to and part of the head structure of *N. gonorrhoeae* pilin (88). The determination of surface regions of the pilin of *P. aeruginosa* will be shown to be important in section 1.IV when the structure of the pilus is discussed.

## III. The Disulfide Loop.

The disulfide loop is a feature of type IV pilins that occurs at or near the C-terminus of the pilin protein. Sastry *et al* (1985) were able to show that the two cysteine residues at positions 129 and 142 of PAK pilin formed an intrachain disulfide bridge in the native protein (109). Cleavage products were analyzed in order to map antigenic determinants of the pilin protein. Since antigenic determinants are generally located on the surface of a protein (3, 4, 118), this approach was used to identify potential surface-exposed regions of the pilin protein and gain more understanding of the structure of pilin. Neither the disulfide linkage nor the cysteines were found to be important antigenic sites on the protein, however, the disulfide loop region (residues 128-144) was moderately immunogenic, leading to the suggestion that it was the amino acids within the loop that were antigenically important (121). This was among the first clues that the disulfide loop had a crucial role to play in the function of *P. aeruginosa* pili. Another clue was discovered when Lee *et al* (1989) demonstrated that antibodies against C-terminal region synthetic peptides unable to form a disulfide bridge, did not recognize either PAK or PAO native pili in ELISA assays (66). This suggested that the disulfide bridge was important for the conformation of the pilin C-terminal region epitope.

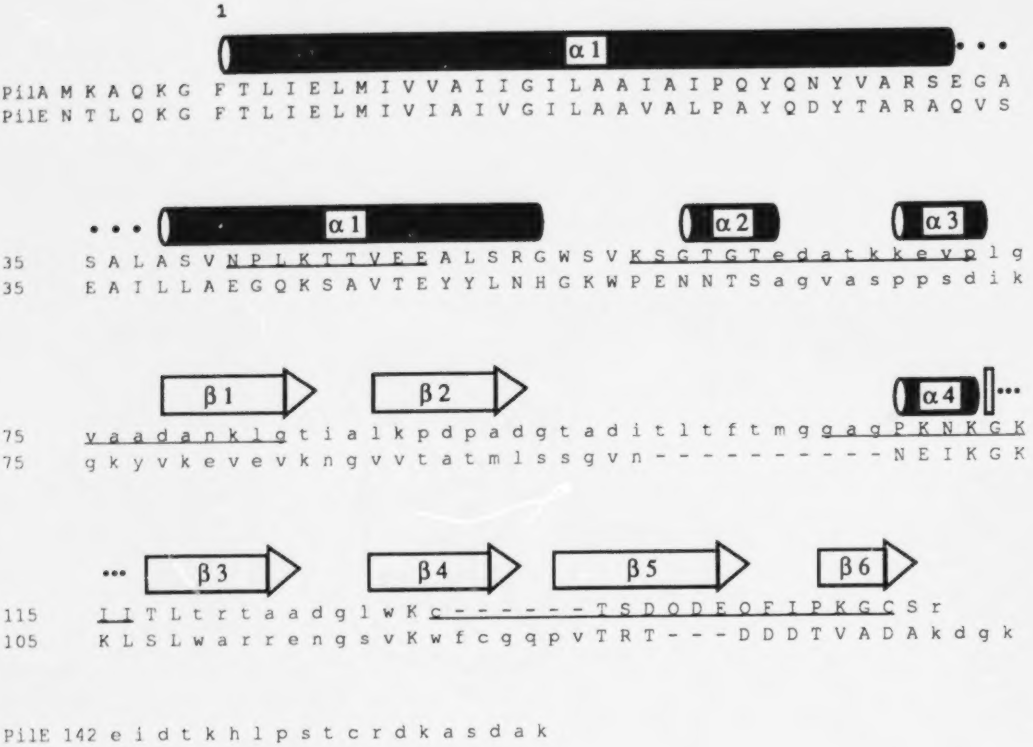

1
α1
PilA M K A Q K G F T L I E L M I V V A I I G I L A A I A I P Q Y Q N Y V A R S E G A
PilE N T L Q K G F T L I E L M I V I A I V G I L A A V A L P A Y Q D Y T A R A Q V S
α1
α2
α3
35 S A L A S V N P L K T T V E E A L S R G W S V K S G T G T e d a t k k e v p l g
35 E A I L L A E G Q K S A V T E Y Y L N H G K W P E N N T S a g v a s p p s d i k
β1
β2
α4
75 v a a d a n k l g t i a l k p d p a d g t a d i t l t f t m g g a g P K N K G K
75 g k y v k e v e v k n g v v t a t m l s s g v n - - - - - - - - - - - N E I K G K
β3
β4
β5
β6
115 I I T L t r t a a d g l w K c - - - - - - T S D Q D E Q F I P K G C S r
105 K L S L w a r r e n g s v K w f c g q p v T R T - - - D D D T V A D A k d g k
PilE 142 e i d t k h l p s t c r d k a s d a k

Fig. 1.1. Comparison of the amino acid sequences of *P. aeruginosa* PAK pilin (PilA) and *N. gonorrhoeae* pilin (PilE). Numbering begins with the first residue of the mature pilin subunit (shown in bold above the sequence) and amino acids before the first residue denote the signal peptide which is cleaved from the mature protein. Capital letters represent regions of highest similarity (116). Underlined residues represent putative surface domains of PAK pilin (85). Domains of the *N. gonorrhoeae* pilin subunit (as deduced from the x-ray crystallographic structure of Parge *et al*) appear above the sequences (88).

Later experiments were aimed at determining the importance of the disulfide loop for pilus function in adherence and bacteriophage adsorption. This structure had been shown to promote adherence to mammalian cell receptors (56, 87). It was also shown to be involved in the adsorption of a pilus-specific bacteriophage to the tip of the PAK pilus when a mutation to one of the residues within the disulfide loop prevented the attachment of the tip-binding phage Pf1 (85). Evidence of the involvement of the disulfide loop in the binding domain of *P. aeruginosa* pili was indirect until the development of a whole cell bacterial attachment assay, as described in this thesis, allowed assessment of the adherence ability of a disulfide loop insertion mutant that retained the ability to assemble pili (33).

## IV. Pilus Assembly, Structure and Function.

Watts *et al* (1982) identified pools of pilin representing 4 to 5% of the membrane protein in the inner and outer membranes of *P. aeruginosa* PAK and a multipiliated mutant of PAK (122). These pools were thought to be the source of pilin subunits for the assembly of pili. It is not known whether this assembly takes place at the cytoplasmic surface or at the cell surface and the signal for pili synthesis is also unknown. What is known is that in the case of cystic fibrosis pulmonary infections, colonizing *P. aeruginosa* undergoes a conversion to mucoidy after the colonization has been accomplished by a piliated form, leading to the conclusion that there must be some environmental signal for pili synthesis (38, 39, 106).

It is clear that the bacteria require a protein transport system to localize the pilin subunits in the membrane for polymerization. Chaperones are likely needed to prevent aggregation of the pilins into secretion-deficient conformations and to keep them in an assembly-competent state (54). The PapD protein serves this function in *E. coli* P-pili synthesis by complexing with the PapG adhesin after translocation of this protein across the cytoplasmic membrane (53, 68). PapD has also been shown to complex with the PapE pilin-like protein prior to pilus assembly (68). Other proteins are undoubtedly required for the assembly and retraction processes. P-pili require PapC, an outer membrane protein essential for the assembly and surface localization of the pili that is thought to help dissociate PapD from the pilin proteins as they are assembled into a pilus (54). Likewise, type IV pili require a secretory and assembly mechanism and this became evident when some of the gene products required for pilus expression were found to share homology with secretory proteins of other bacteria (49, 124). This also indicated that the pilus assembly mechanism did not involve the proteins of the general secretory pathway, but rather made use of a Sec-independent pathway (49, 97, 124). The signal sequence of *P. aeruginosa* prepilin resembles the signal sequences of other secretory proteins except that in the case of *P.*

*aeruginosa* pilin, the PilD prepilin peptidase processes this sequence at an unusual site that releases only a short 6-residue peptide. This first processing step involves two critical prepilin residues: the glycine at -1, required for cleavage and assembly, and the glutamate at +5, required for assembly and methylation, but not cleavage (62, 71, 91, 96, 115).

Following the translocation of the pilin subunits across the cytoplasmic membrane, assembly into a pilus structure takes place. It is not yet known how many gene products are involved in this process or how it occurs but some new light is being shed on the nature of the signal transduction system involved in pilus assembly (24, 25, 26). Chapter 4 of this thesis details the characterization of a spontaneous mutant in the *pilG* gene. PilG is a homologue of the CheY protein which functions in chemotaxis in enteric bacteria (24). Evidence is provided that PilG may serve a similar function in the regulation of pilus assembly in *P. aeruginosa.*

The techniques of X-ray fibre diffraction and analysis of diffraction patterns were used to investigate the structure of pili from *P. aeruginosa* PAK and PAO (35). Hydrodynamic data, in addition to the X-ray diffraction information, allowed a model for pilus structure to be proposed. Fig. 1.2 shows the model of Watts *et al* (1983) (120). This helical structure is a based on a molecular weight of pilin of approximately 15,000 kDa, which gives 5 pilin subunits per turn of the helix. The helix is 52 Å in diameter with a central channel of 12 Å and a pitch of 41 Å. The pilus fibres can vary in length from 0.5 to 10 μm with an average length of approximately 1 to 3.5 μm (16, 84). Recent work by Parge *et al* (1995) supports and expands on this structure of the pilus. These investigators obtained the crystallographic structure of *N. gonorrhoeae* type IV pilin and from that information were able to predict a pilus structure that conforms very well to the measurements of the *P.aeruginosa* pilus. They predicted a right handed helix containing five pilin residues per turn in which the $\alpha_1$-helices of the pilins were approximately parallel to the fibre axis with the pilin β-sheet structures wrapping around the core of packed $\alpha_1$-helices (88). Packing of the $\alpha_1$-helices was aided by the predicted interaction of the positively charged N-terminus of subunit$^{N+1}$ with the conserved, negatively charged Glu 5 of subunit$^{N}$. This model helix had a diameter of 60 Å and a pitch of 41 Å, almost identical to the measurements for *P. aeruginosa* pili. Because of the ability of *P. aeruginosa* to assemble pili from *N. gonorrhoeae* pilin subunits (51), it is reasonable to suppose that the structure proposed for *N. gonorrhoeae* pili is similar to that of *P. aeruginosa.* One difference that was observed between the pilus structures of the two organisms was the location of the disulfide loop (DSL) structures of the pilin subunits. In the *N. gonorrhoeae* pilus structure, the DSL of each pilin molecule is exposed on the side of the pilus as part of the helical array of subunits (88). In *P. aeruginosa*, monoclonal antibodies specific for the DSL bind to the pilus only at the distal tip, not along the sides of the pilus, leading to the conclusion that the DSL structure is exposed only at the tip of this pilus, and is otherwise sequestered as the pilin subunits form the pilus helix (67).

### V. Sensitivity to Pilus-Specific Bacteriophages and Twitching Motility.

D. E. Bradley described bacteriophages (phages) that required functional pili for infection of *P. aeruginosa* . These phages were observed by transmission electron microscopy to be bound to the pilus and appeared to require retraction of the pilus to enter the bacterial cell and replicate (14, 15, 17, 18, 20). Subsequently, other bacteriophages were found to be pilus-specific and these became important tools for the isolation and characterization of bacterial mutants in pilus biogenesis. Resistance to one or more pilus-specific phages became a standard method for isolating mutants blocked in various steps of pilus biogenesis. Most of these mutants had been observed to be devoid of pili, indicating that the mutation conferring phage resistance resulted in an inability to assemble pili (20, 24, 25). There were a few mutants, however, that produced pili able to adsorb phage but the cells were not able to be infected by the phages. This was suggested to be due to the pili being non-retractile such that the phages could not get close to the cell surface to accomplish infection (20).

The ability to retract the pili also gives rise to a form of locomotion called twitching motility which provides bacteria with a means of spreading across a solid surface (19, 43, 44). This form of motility is independent of flagella, which require a certain volume of liquid in order to impart motility and it is visualized as a spreading zone at the outer edge of a bacterial colony. Wild type *P. aeruginosa* colonies are large and have a rough, almost dry appearance with a ruffled edge compared to twitching motility mutants whose colonies are smaller, domed, and smooth. Although the exact mechanism of this form of motility remains uncharacterized, Bradley was able to show that retractile pili were required since twitching motility was absent in organisms unable to produce pili or in organisms expressing non-retractile pili (19). He also demonstrated that the presence or absence of flagella had no effect on the appearance of a zone of twitching motility at the outer edge of *P. aeruginosa* PAK colonies. Some of the more recent information about twitching motility and pilin expression has come from the identification of a group of genes that appear to function in a twitching motility signal transduction network that resembles the organization

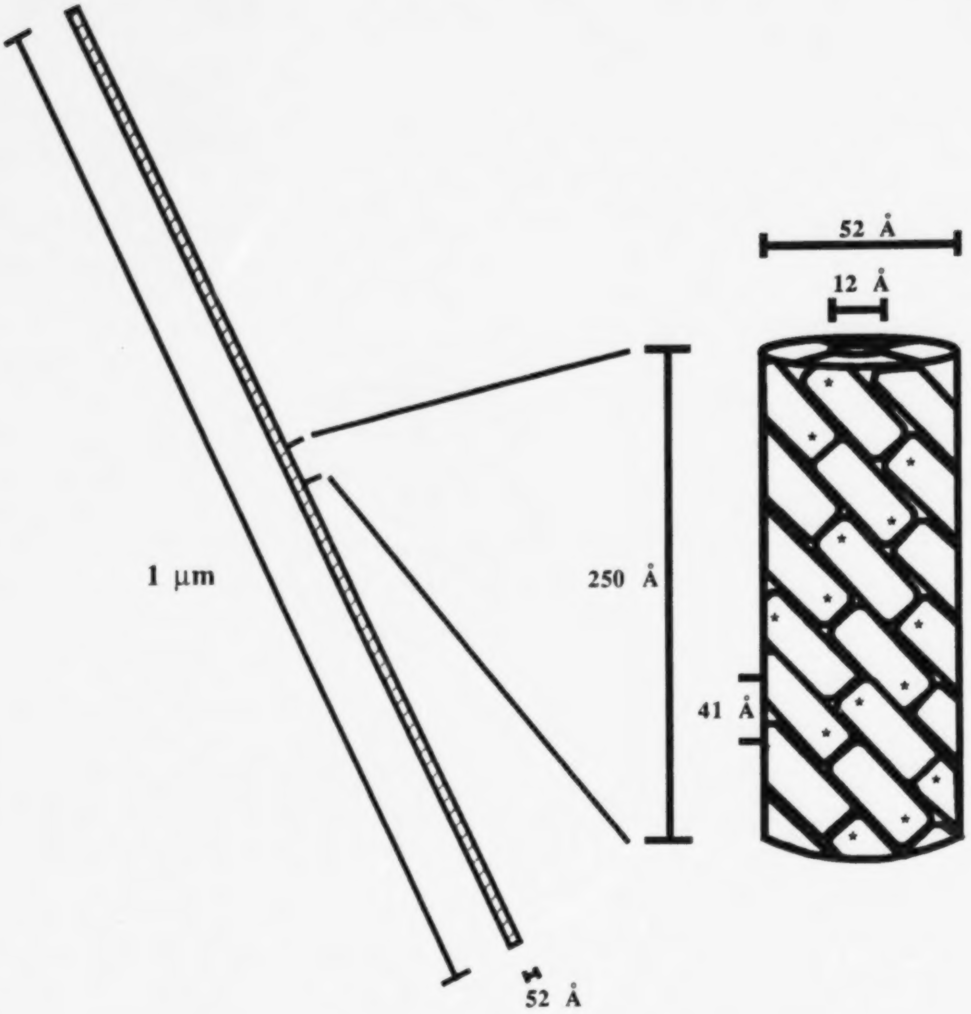


Fig. 1.2. A model for the structure of *P. aeruginosa* PAK pilus structure based on X-ray fiber diffraction and hydrodynamic studies. This model was proposed by Watts *et al* (1983) (120). An approximately 250 Å section of the pilus is enlarged to show a possible pilin subunit orientation. The stars represent one end of the pilin subunit. This structure allows 5 pilin subunits per turn of the helix, which is 52 Å in diameter with a central channel of 12 Å and a pitch of 41 Å. The pilus fibres have an average length of approximately 1 to 3.5 μm (16).

of the enteric chemotaxis system (24, 25, 26). This system is discussed in more detail in section 1.IV and Chapter 4.

## VI. Genetics of Pili Expression.

The assembly of pili in *P. aeruginosa* is a complex process involving a number of gene products with most of our information about these gene products being discovered in the last five years. It has been estimated that there may be up to 40 or more genes involved in the biosynthesis and expression of type 4 pili in *P. aeruginosa* (48, 49). The chromosomal organization of the currently known pili-associated genes is shown in Fig. 1.3. These include not only genes for the pilin structural subunit and products for its maturation but also those for its assembly into mature pili. The required genes can be subdivided into a number of functional groups:

a) The structural gene plus genes involved in the maturation and transport of the pilin protein (*pilA* and *pilD*) (81, 90, 109).
b) Genes involved in the assembly of the mature pilus structure (*pilB, pilC, pilE, pilQ, pilT, pilM* and *pilV*) (2, 49, 72, 73, 116).
c) Genes that appear to function in transduction of the environmental signal for pilus expression (*pilG, pilH, pilI, pilJ* and *pilK*) (24, 25, 26).
d) Genes controlling the expression of other genes involved in pilus biosynthesis (*rpoN, pilR* and *pilS*) (58, 59, 72).
e) Genes that affect the assembly of pili but have not yet been well characterized (*pilN, pilO, pilP, pilU* and *pilZ*) (1, 73, 105).

To date, 22 genes have been identified that function in pilus biogenesis and many of these have yet to be further characterized to determine their specific role. Fig. 1.4 is a schematic diagram outlining the possible functions of many of the pilus assembly proteins. PilA is the structural subunit of the pilus. Transcription of the single copy *pilA* gene requires the PilS, PilR, and RpoN proteins (13, 58, 60, 90). DNA sequence data suggested that the pilin promoter is recognized by RNA polymerase holoenzyme with the alternative sigma factor RpoN. Also, *P. aeruginosa* RpoN was demonstrated to activate RpoN-dependent transcription in an *E. coli* mutant lacking RpoN (58). In the general model for RpoN-dependent transcription, a specific transcriptional activator is required in addition to RpoN for significant transcription from a gene with an RpoN-dependent promoter (41, 47, 55, 104). Consistent with this, the cloned *P. aeruginosa* pilin gene was expressed poorly in *E. coli*, even in the presence of a functional RpoN (114), indicating that transcription of *pilA* also required one or more regulatory proteins not present in *E. coli* (58). PilR was found

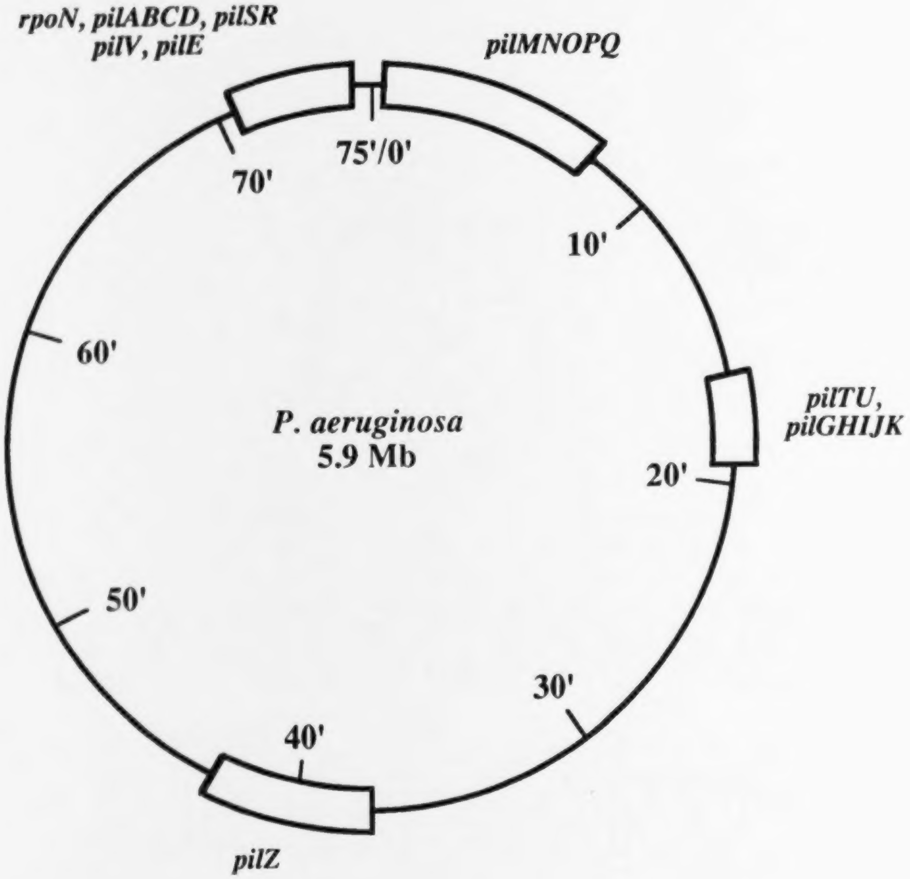

rpoN, pilABCD, pilSR
pilV, pilE
pilMNOPQ
75'/0'
70'
10'
60'
P. aeruginosa
5.9 Mb
pilTU,
pilGHIJK
20'
50'
30'
40'
pilZ

Fig. 1.3. Circular map of the *P. aeruginosa* PAO1 chromosome showing the approximate locations of genes involved in pilus biogenesis. The start position of the 75-min (75') map is at *ilv* (1, 34).

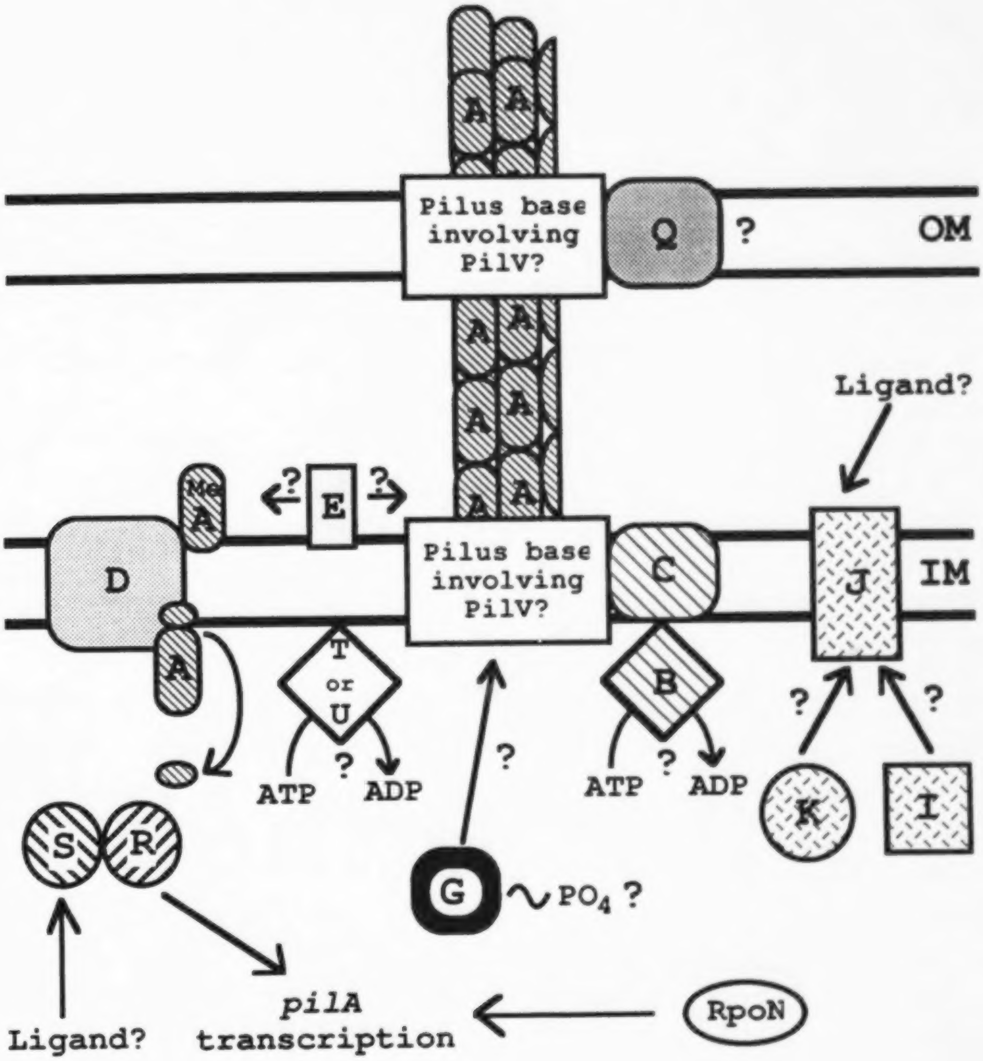

A
A
Pilus base involving PilV?
Q
?
OM
A
A
A
A
Ligand?
Me
A
?
E
?
A
D
Pilus base involving PilV?
C
J
IM
A
T or U
B
?
?
?
ATP
ADP
?
ATP
ADP
K
I
S
R
G
PO4 ?
Ligand?
pilA transcription
RpoN

Fig. 1.4. Schematic representation of some of the gene products involved in pilus biogenesis (see text for explanation and references). Abbreviations: A, PilA; B, PilB; C, PilC; D, PilD; E, PilE; G, PilG; I, PilI; J, PilJ; K, PilK; Q, PilQ; R, PilR; S, PilS; T, PilT; U, PilU; V, PilV; IM, inner membrane; OM, outer membrane; Me, methyl; $PO_4$, phosphate. Question marks indicate unknown functions or interactions. Gene products not included in this representation are: PilH, PilM, PilN, PilO, PilP and PilZ. Relative sizes of symbols are not intended to indicate relative sizes of the gene products.

to activate transcription of the *pilA* gene in *P. aeruginosa* and was shown to share homology with the two putative nucleotide-binding site regions of NtrC from *Rhizobium meliloti*. PilR also contained the invariant Asp-11, Asp54, and Lys-109 of this family of transcriptional regulators (59). Like NtrC, PilR is probably phosphorylated at Asp-54 or Asp-11, allowing the activator to facilitate an open-promoter complex formation with RpoN-containing RNA polymerase (59), possibly by binding to a regulatory site upstream of *pilA* (89) and then interacting with the RpoN-RNA polymerase through a DNA looping mechanism (48).

The subsequent discovery of the PilS sensor protein led to the suggestion that PilS and PilR belonged to the two-component (sensor-regulator) family of environmentally responsive transcriptional activators (13, 48). The C-terminus of PilS was shown to be homologous to the kinase domain in other sensor proteins such as NtrB of *Klebsiella pneumoniae*, but the N-terminus did not show homology to any other known sensor sequences, indicating a novel specificity for an as yet, unknown cytoplasmic signal (13, 48).

PilA is synthesized as a prepilin precursor containing a short, positively charged signal sequence that is cleaved by the PilD protein (80). Proteins destined for export usually contain a signal sequence of 15 to 30 residues, composed of one or more positively charged residues at the N-terminus, followed by a hydrophobic region and a cleavage site which is slightly polar (78, 131). Prepilin contains such a sequence but is unique in that the cleavage site is between the positively charged sequence and the hydrophobic region (91). Subsequent to signal peptide cleavage, the first residue of the mature pilin (usually a phenylalanine or methionine) is methylated by PilD. An invariant glutamate at the +5 position from the cleavage site is required for methylation but not for prepilin processing since substitutions at this residue had no effect on processing but the pilin was not methylated and the subunits were not assembled into pili (91, 115). It is the processed, methylated PilA that is assembled into a pilus.

The deduced amino acid sequence from the *pilE* gene was observed to share sequence identity with the N-termini of type IV prepilin proteins. The PilE N-terminus contains a 7-residue basic leader sequence followed by a consensus cleavage site for PilD (preliminary evidence was obtained for the processing of PilE by the PilD prepilin peptidase-transmethylase) and then a hydrophobic region including the residues Tyr-24 and Tyr-27 which have been implicated in PilA subunit-subunit interactions (105, 120). These results indicated that PilE was probably localized to the membrane and several hypotheses have been suggested to account for the pilus-less phenotype of *pilE* mutants (105). One hypothesis was that PilE could be a minor pilin protein, although this is unlikely since no subunit other than PilA has been detected in *P. aeruginosa* pili (92, 105). A second hypothesis was that PilE functions as an anchor protein much like the PapH protein of the Pap pilus in *E. coli*. PapH is located at the base of the Pap pilus, serving as a signal that controls pilus length and mutants lacking PapH release unusually long pilus filaments into the medium (54). This also seems an unlikely role for PilE since mutants lacking this protein also lack pili (105). A third, and more likely hypothesis, is that PilE performs a chaperone-like function, perhaps interacting with processed PilA subunits and preventing their aggregation prior to assembly. Russell and Darzins suggested that PilE may also serve as a pilot that guides PilA subunits to and from the assembly platform during pilus polymerization and retraction, similar to the PapD chaperone of the P pilus system (65, 68, 105).

The PilV protein also shares considerable (37% identity, 69% similarity) homology with the PilA subunit at its N-terminus. PilV contains one of the tyrosine residues thought to be important for pilin subunit-subunit interactions; Tyr-30 of PilV corresponds to Tyr-24 of PilA in a sequence alignment by Alm and Mattick (2). An alignment of PilV with the *P. aeruginosa* XcpV (functions in extracellular protein secretion) revealed that PilV also shared 57% similarity with the N-terminal domain of XcpV (2). XcpT,-U,-V, and -W were suggested to interact as a multicomponent structure in a helical array related to that of pili, providing the framework for a possible cell wall continuity (7, 49). Alm and Mattick's suggestion for PilV function was that it (and also PilE) might be a component of a similar structure which would act as a base for the projection of a PilA filament. This is possible given that complementation of a *pilV* mutation with a cloned *pilV* gene under control of a promoter weaker than the *lac* promoter conferred bacteriophage sensitivity but not twitching motility or pilus assembly to the non-piliated, phage resistant mutant. This was explained by postulating that the small amount of PilV produced from the weak promoter allowed sufficient PilA to be translocated to the cell surface in order to confer the bacteriophage-sensitive phenotype but not enough PilV was present to allow functional pili to be assembled (2).

Five more gene products that affect the expression of fully functional pili at the level of assembly are PilB, PilC, PilT, PilU and PilZ. The PilB, PilT and PilU proteins share significant amino acid sequence similarity across their entire sequences with two highly conserved regions: a highly conserved nucleotide-binding domain present in many prokaryotes and eukaryotes (Walker box A), and a less well-conserved secondary domain present in only some nucleotide-binding proteins (Walker box B) (107, 119, 124). The indication is that these proteins are probably cytoplasmic nucleotide-binding proteins responsible for providing energy for subunit translocation or assembly and it has been proposed that this family of proteins may be associated with the inner membrane (80, 95, 116, 124). Note that the nucleotide shown with these proteins in Fig. 1.4 (ATP), may or may not interact with PilB or PilT as this has not been determined experimentally. One difference observed between PilB, PilT and PilU mutants was that PilB mutants were unable to synthesize pili whereas PilT and PilU mutants assembled pili but were incapable of twitching motility. Another interesting difference between PilT and PilU was that PilT mutants acquired resistance to the pilus-specific bacteriophage PO4 whereas PilU did not. This led Whitchurch *et al* to suggest that perhaps PilB and PilT act antagonistically with PilB involved in incorporation of pilin into pili and PilT involved in the disassembly of the pilus (124). The differences between the phenotypes of PilT and PilU mutants has also called into question the accepted notion that functional pili are required for pilus-specific phage sensitivity. The fact that PilT and PilU mutants are both hyperpiliated and incapable of twitching motility, yet PilU mutants remain sensitive to pilus-specific bacteriophages, indicates that it is possible that the absence of twitching motility is not the result of an inability to retract the pili or that retraction is not required for phage infection. Whitchurch and Mattick also suggested that pilus-specific phages may utilize some of the components of the system involved in the assembly and function of pili but that there seems to be no consistent relationship between the presence or absence of pili, twitching motility, and phage sensitivity (125). As this is a complex system, more information about the exact roles and interplay of factors involved in pilus assembly will be needed before this relationship is fully understood.

Very little detailed information has been published about the PilC or the PilZ proteins. PilC is required for pilus assembly in *P. aeruginosa* and its deduced amino acid sequence suggests an integral cytoplasmic membrane protein. Mutants in *pilC* express pilin that is in the fully processed form, so that the defect in these mutants occurs in the assembly process (80, 116). PilZ is also required for pilus assembly as mutants lacking this protein have the normal processed pilin pools in the membranes, but pili are not assembled. PilZ shows no homology to any other proteins in the sequence databases but on the basis of its predicted amino acid sequence and the corresponding Kyte-Doolittle hydropathy plot, it is probably a cytoplasmic protein that may associate with the inner membrane (1).

*P. aeruginosa* mutants lacking PilQ were unable to synthesize pili and resistant to pilus-specific bacteriophages. The N-terminus of the deduced amino acid sequence of PilQ contained a short positively charged region followed by a stretch of 17 mostly hydrophobic amino acids, characteristic of the signal sequence of secretory proteins and sequence similarities to known outer membrane proteins suggested an outer membrane location for PilQ (72). PilQ's shared amino acid sequence homology to the PulD family of outer membrane proteins was concentrated in the C-terminus of the protein, involving a region containing a secretion protein motif proposed to be involved in outer membrane localization (52, 72, 117). PilQ shared highest overall homology with the *N. gonorrhoeae* Omc protein, an outer membrane protein that forms high molecular weight complexes in the outer membrane as discussed below with the PilM protein (117). The suggestion is that PilQ may be involved in the formation of a structure at the outer membrane through which a pilus could pass (72, 73). The unknown nature of such a structure is indicated in Fig. 1.4 by the presence of only one representation for PilQ since, if it does function as a multimer, the number of subunits and the nature of their possible interaction with the pilus has not yet been investigated.

Upstream of the *pilQ* gene is a cluster of four genes, also required for pilus biogenesis. These genes, *pilM*, *pilN*, *pilO*, and *pilP*, appear to form an operon and be expressed from a promoter upstream of *pilM*. The deduced amino acid sequence of PilM indicated that it was largely hydrophilic and appeared, therefore, to be a cytoplasmic protein (73). It also shared limited sequence similarity with the cell division protein FtsA of *E. coli* and *Bacillus subtilis*, in a domain discovered to be involved in ATP binding (8, 12, 103). PilM and PilQ were proposed to be involved in the formation of multimeric structures since the transformation of *pilM* and *pilQ* deletion mutants into wild-type cells resulted in a dominant negative phenotype (73). The hydropathy profile of PilN indicated that this protein had one extended hydrophobic region that could function as a cytoplasmic membrane anchor, although it was not observed to contain a typical signal sequence. PilN also shared a low level of amino acid sequence similarity with XpsL from *Xanthomonas campestris*, a protein required for protein secretion (73). Like PilN, PilO did not have a typical signal sequence but it was predicted to contain a long stretch of hydrophobic amino acids that might serve to localize it to the cytoplasmic membrane. Little other information about PilO was available as it did not share any significant amino acid sequence homology with any other proteins in the sequence databases (73). PilP contained a signal sequence characteristic of lipoproteins: a short, positively charged N-terminus followed by a hydrophobic domain and ending with a cysteine residue at the cleavage site. Based on this information, Martin *et al* proposed that PilP was likely to be exported to the periplasm and acylated (73). No homologues of PilP were found in the sequence databases, although it is interesting to note that the pullulanase secretion system of *K. oxytoca* requires the PulS lipoprotein, which is proposed to interact with the outer membrane protein PulD, which is homologous to PilQ (73, 97, 130).

The final group of gene products to consider all share homology with various members of the enteric chemotaxis signal transduction network or that of the gliding bacterium *Myxococcus xanthus*. The *pilG, -H, -I, -J* and *-K* genes occur together in a cluster close to the *pilT* locus on the *P. aeruginosa* chromosome (24, 25, 26). PilG shares considerable amino acid sequence homology with the CheY protein of the enteric chemotaxis system (24). CheY is a well-characterized member of a family of single-domain response regulator proteins responsible for switching the direction of flagellar rotation to induce tumble responses (112, 113). Darzins proposed that the PilG protein may have a similar function in pilus expression in *P. aeruginosa* by participating in a signal transduction cascade (24). The possible role of PilG is discussed in more detail in Chapter 4. The PilH protein also shared considerable amino acid sequence homology with CheY (and PilG) but is likely to play a different role than PilG since a mutant lacking PilG was incapable of twitching motility and broadly resistant to pilus-specific bacteriophages unlike a *pilH* mutant which was piliated, phage-sensitive, and capable of an altered form of twitching motility giving rise to circular swirls at the outer edge of the moving zone of a colony (25). This phenotype is similar to a motility pattern demonstrated by *frz* mutants in *M. xanthus* at low cell density, that rarely reverse their direction of gliding (129). This led Darzins to propose that PilH may play a role in controlling the direction of twitching motility. PilI shared homology with FrzA, a *M. xanthus* CheW homologue and PilJ shared homology with the C-terminal highly conserved domain of Tsr, the serine receptor, of the enteric chemotaxis system. Tsr is one of the methyl-accepting chemotaxis proteins (MCPs) that can form a ternary complex with CheW and CheA which leads to the activation of CheY (the interplay of factors in the enteric chemotaxis system is explained in more detail in Chapter 4.A.II). The highly conserved domain of Tsr is conserved in most if not all MCPs and has been suggested to play a role in interaction with CheW, while the periplasmic N-terminal domain is responsible for ligand binding (69). It is possible that PilI and PilJ may interact in a similar manner as part of a chemotaxis system regulating twitching motility and the expression of pili in *P. aeruginosa* (25). The *pilK* gene also codes for a chemotaxis system homologue as PilK shares 32% identity with the CheR methyltransferase protein that participates in the adaptive response in enteric chemotaxis (79). The exact role of PilK in pilus assembly awaits further analysis.

The assembly system for type IV pili seems to have remained highly conserved since it is possible to assemble heterologous pili in *P. aeruginosa* utilizing cloned pilin genes from other species (9, 13, 30, 31, 74). It appears that the Pil proteins involved in pilin subunit transport and processing did not evolve specifically for pilus assembly since genes such as *pilB*, *pilC*, *pilD*, and *pilT* have homologues in other bacteria that do not produce type IV pili. Instead, it seems that these Pil proteins are members of a class of proteins functioning in a protein transport mechanism that has been adapted to a variety of bacterial systems (124). Another intriguing aspect of pilus assembly that has not yet been investigated is the number of gene products involved in apparent interactions with the pilin subunits or the pilus itself (PilE, PilV, PilC). It is possible that some of these proteins may interact in the manner of the XcpT, -U, -V and -W proteins to form a channel bridging the inner and outer membranes of *P. aeruginosa*. If a specific structure such as this was required for pilus assembly, that would explain why a mutation in any one of these genes results in the absence of pili but normal processing of pilin subunits. The picture of pilus assembly that is emerging from the information now available is one that involves environmental control of pilus assembly in two ways. The first is a system controlling the actual sythesis of pilin subunits, involving the PilS-PilR sensor-regulator pair and RpoN control of transcription of the *pilA* gene. The second system involves a separate, chemotaxis-like control of pili assembly and function through the PilG, -H, -I, -J, and -K proteins, with pilus assembly occurring via a pathway related to a number of protein-secretion and cell surface complex-forming pathways (73).

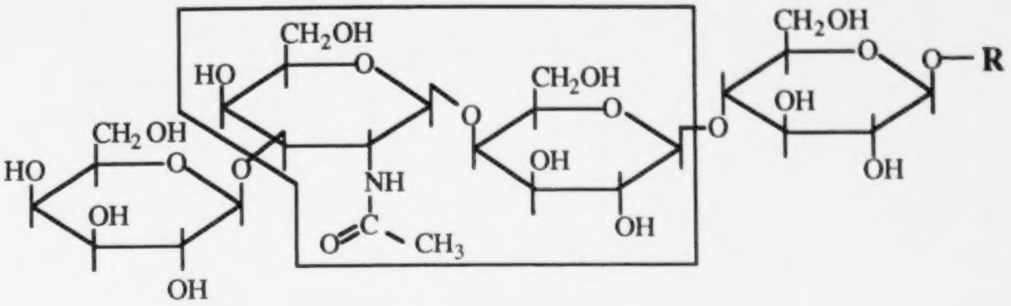
### VII. *Pseudomonas aeruginosa* Adherence.

Falkow *et al* defined a microbial pathogen as any microorganism that has the capacity to cause disease within a particular host and they also described the necessity of entering a host and finding a suitable niche as a fundamental attribute of bacterial pathogenicity (32). Further, they stated that the first specific interaction between a pathogenic microorganism and its host involves attachment of the pathogen to a eukaryotic cell surface. Bacterial pili are a common structure employed to accomplish this adherence and this discussion will be restricted to the role of pili in the attachment of *P. aeruginosa* to host cells.

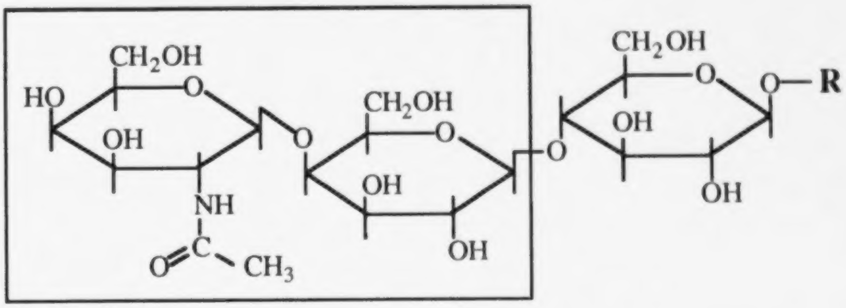
Woods *et al* first suggested that pili could mediate the adherence of non-mucoid *P. aeruginosa* to human buccal epithelial cells (BECs) in 1980 (128). These authors found that heat- or formalin-killed *P. aeruginosa* could not adhere to BECs whereas bacteria killed by ultraviolet light retained their adherence ability. They attributed this to the loss of pili after heat or formalin treatments and retention of pili after ultraviolet light treatment, as observed by electron microscopy. Furthermore, enzymatic digestion of pili also resulted in a decreased adherence ability as did preincubation of BECs with purified pili (128). In 1986, Paranchych *et al* observed that both purified whole pili and anti-pilus antiserum could block the interaction of *P. aeruginosa* with human buccal endothelial cells and polymorphonuclear leukocytes, leading them to identify pili as the epithelial cell adherence factor (87). These researchers also observed that a peptide fragment encompassing 23 amino acid residues at the C-terminus of the pilin protein was able to inhibit *in vitro* adherence of the bacteria to buccal cells, suggesting that the C-terminal domain of the pilin protein contained the receptor-binding function. The C-terminal disulfide bridge was also demonstrated to be important for adherence when synthetic peptides, consisting of the last 29 and 17 amino acids of the C-terminus of *P. aeruginosa* PAK pilin, were shown to bind to BECs more effectively when in the oxidized state (29, 56).

Research aimed at the identification of the epithelial cell receptor(s) for pili indicated that PAK pili bound to five major glycoproteins from the BEC membrane and this binding could be inhibited by sialic acid and L-fucose (28). Previous work had indicated that the sugar moieties sialic acid, asialo-$G_{M1}$ (gangliotetraosylceramide), and L-fucose were likely important as *P. aeruginosa* receptors, but these experiments did not distinguish between different adhesins, such as pili or alginate, precluding the identification of the receptor-adhesin system involved (27, 63, 75, 98, 99). Krivan *et al* demonstrated that *P. aeruginosa* and *P. cepacia* isolated from cystic fibrosis patients bound specifically to asialo-$G_{M1}$ and asialo-$G_{M2}$ (gangliotriaosylceramide) by thin-layer chromatography (63). They also found that a number of pulmonary pathogens, including the three classic infectious bacteria involved in cystic fibrosis (*P. aeruginosa, Haemophilus influenza,* and *Staphylococcus aureus*), bound specifically to fucosylasialo-$G_{M1}$, asialo-$G_{M1}$, and asialo-$G_{M2}$, which all contain the carbohydrate sequence GalNAcβ1-4Gal (64). These structures are shown in Fig. 1.5. Hazlett *et al* were able to demonstrate that treatment of mouse corneal epithelium with a protease-producing strain of *P. aeruginosa* or trypsin could unmask asialo-$G_{M1}$ that was not normally accessible in an uninjured cornea (42). They also showed that antibodies to asialo-$G_{M1}$ could inhibit *P. aeruginosa* binding to mouse corneas, providing more evidence for the role of asialo-$G_{M1}$ as a receptor for *P. aeruginosa* in an *in vitro* bacterial binding assay. The biological relevance of bacterial binding to asialo-$G_{M1}$ was suggested by the identification of this glycosphingolipid in human lung

**Asialo-G$_{M1}$** Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer



**Asialo-G$_{M2}$** GalNAcβ1-4Galβ1-4Glcβ1-1Cer



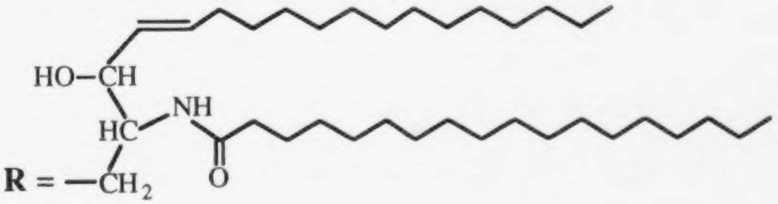


Fig. 1.5. Glycolipid structures with possible involvement as cell surface receptors for *P. aeruginosa* pili binding. Purified *P. aeruginosa* PAK and PAO pili were shown to bind specifically to asialo-G$_{M1}$, asialo-G$_{M2}$, and GalNAcβ1-4Gal (67, 111). Boxed structures are GalNAcβ1-4Gal and **R** is the ceramide portion of the asialo-G$_{M1}$ and asialo-G$_{M2}$ structures. Abbreviations: Cer, ceramide; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose.

extracts (64). Finally, purified *P. aeruginosa* PAK and PAO pili were shown to bind specifically to asialo-$G_{M1}$, asialo-$G_{M2}$, and GalNAcβ1-4Gal (67, 111). This binding was demonstrated to be a pilus tip-associated event and was blocked by synthetic peptides encompassing the C-terminal disulfide loop domain of the pilin subunits for each of PAK and PAO, indicating that the pilus adherence phenomenon involves a tip-exposed C-terminal region of the pilin subunit.

Bacterial whole-cell adherence assays involving *P. aeruginosa* and BECs allowed the pilus-binding systems of different strains to be characterized through the calculation of association and dissociation constants (29, 57). These assays involved detecting the number of radio-labeled bacteria bound to the surface of the BECs. An advantage of this method was that the bacteria had a high specific activity due to labeling with $^{35}S$. This made the bound bacteria easy to quantitate with good reproducibility. While the radioactive label was specific, however, it was also more difficult to use because of containment requirements. Another limitation of this assay was the fact that only 5% of the BECs were viable at the time of the assay (57). Bacteria were presented with a more consistent cell surface when adherence assays were developed using monolayers of human lung pneumocytes in which the epithelial cells were 95% viable (23, 33). The method of bacterial enumeration for this assay was to stain the monolayers and bacterial cells and then count the numbers of bacteria observed under the light microsope. This method also had the advantage of good reproducibility but was time consuming and did not differentiate between viable and non-viable bacteria. Chapter 3 of this thesis describes in more detail the development and experimental use of an adherence assay that attempted to address some of these concerns by making use of a viable monolayer of human lung cells and a plate count method of bacterial enumeration. This allowed further characterization of the nature of pili as adherence factors in *P. aeruginosa*.

### VIII. *Pseudomonas aeruginosa* and Cystic Fibrosis.

Cystic fibrosis (CF), first described as a clinical syndrome in 1938, is usually characterized by exocrine pancreatic insufficiency (requiring pancreatic enzyme supplements), an increase in the sweat $Cl^-$ concentration, male infertility, and bronchopulmonary infection. This airway infection leads to the progressive pulmonary deterioration which is responsible for 95% of CF mortality (40, 123). The gene responsible for the CF defect has been identified (102) and the cystic fibrosis transmembrane conductance regulator (CFTR) functions as a cAMP-activated chloride channel (45). CFTR is predicted to consist of five domains: two membrane-spanning domains, two nucleotide-binding domains, and a unique regulatory (R) domain (Fig. 1.6). Mutations in different domains can give rise to different cellular phenotypes ranging from reduced ion flow to incorrect cellular location or reduced amounts of CFTR (123). This is a low conductance channel whose activity is stimulated by cAMP as phosphorylation of the regulatory domain is required for the channel to open (45, 123). In cells affected by the CF mutations, an abnormality in $Cl^-$ ion transport leads to an altered composition of epithelial cell secretions due to the altered movement of water in and out of the cells. The movement of water depends upon the movement of ions across the cells and fluid secretion and reabsorption are controlled primarily by opening of apical membrane $Cl^-$ channels and basolateral $K^+$ channels (76, 77, 93).

It is not known exactly how the altered epithelial cell secretions associated with the CF lung contribute to chronic bacterial colonization. The lungs of neonates may be histologically normal, but their susceptibility to pulmonary infections soon becomes obvious. Infections are typically localized in the major and minor airways rather than the alveoli and these can involve bacteria, viruses, and occasionally fungi, but lead eventually to chronic bacterial colonization. The respiratory pathogens most commonly associated with CF are *S. aureus*, non-capsulate *H. influenzae,* and *P. aeruginosa*. Generally, microbial colonization of the lung tends to follow a sequence during the lifetime of a patient. *S. aureus* usually begins colonization in infancy, followed by *H. influenzae* in childhood, and *P. aeruginosa* in early adolescence, with the highest incidence of *Burkholderia* (formerly *Pseudomonas*) *cepacia* and mycobacteria in the late teens. The development of improved therapies for CF patients has introduced a new problem: an increased spectrum of opportunistic microbes. The increasing incidence of *B. cepacia* and mycobacteria among older CF patients has caused new concerns (40).

*P. aeruginosa* is an accomplished opportunistic pathogen, causing a wide range of infections in compromised patients (11, 40, 61, 94). Its isolation from a number of varied environments, its ability to survive in water, and its resistance to many disinfectants and bacteriostatic agents may contribute to a reservoir of organisms not easily eliminated (11). Generally, colonization of the CF lung by *P. aeruginosa* begins with non-mucoid forms and a conversion to a mucoid variant can occur in less than three months (39, 40). Most CF patients will be colonized with a single strain of *P. aeruginosa* that undergoes phenotypic variation over time but some patients can harbor more than one strain, at least transiently. The sites of first colonization and the mechanism of the colonization are unknown. When adherence of *P. aeruginosa* to buccal epithelial cells was demonstrated

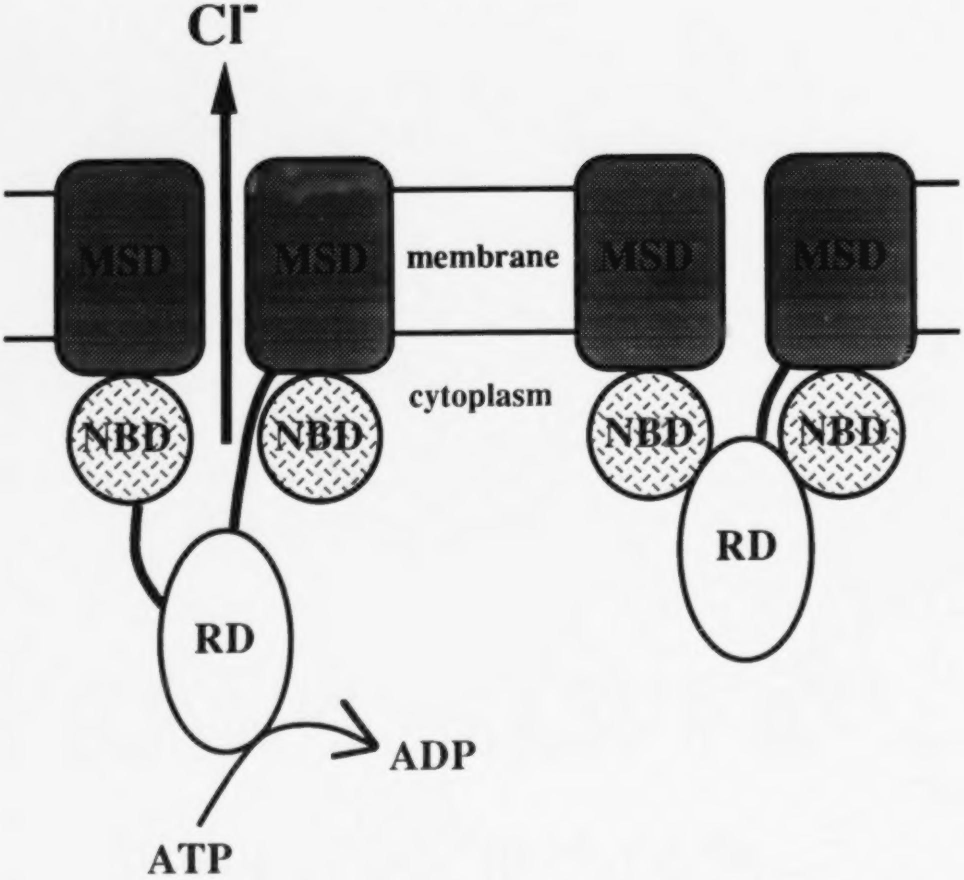

Cl⁻
MSD
MSD
membrane
MSD
MSD
cytoplasm
NBD
NBD
NBD
NBD
RD
RD
ADP
ATP

Fig. 1.6. Schematic diagram of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR consists of five domains: two membrane-spanning domains (MSD), two nucleotide-binding domains (NBD), and a regulatory domain (RD). Phosphorylation of the RD domain by protein kinase A is required for opening the channel and allowing movement of chloride ion ($Cl^-$) across the membrane (45).

*in vitro*, it was suggested that this could be a possible initial site for colonization, but another study was unable to confirm colonization of buccal cells in CF patients (39, 127). Adherence of *P. aeruginosa* to tracheal cells and mucins has also been implicated in CF pulmonary colonization leading to suggestions of alginate, pili, lectins, and exoenzyme S as adhesins (5, 6, 29, 101). More recent work by Farinha *et al* (see Chapter 3) has demonstrated the importance of pili as the primary non-mucoid *P. aeruginosa* adhesin in an *in vitro* A549 human lung pneumocyte attachment assay (33).

Colonization of the CF lung may be aided by the fact that in CF patients, mucus secretions are viscous and mucociliary clearance is impaired. Viral and mycoplasmal infections may damage the mucociliary system and *P. aeruginosa* rhamnolipid, pyocyanin and proteases may inactivate the ciliary beat (40, 46, 126). In addition, *P. aeruginosa* is capable of producing a wide array of virulence factors that damage host tissues and aid in immune system avoidance including alginate, extracellular proteases, exotoxin A, hemolysin, lecithinase, phospholipase, and a leukocidin (70, 94). *P. aeruginosa* is also able to associate with CF mucosal secretions which may help protect the organism from opsonophagocytic killing and contribute to its persistence in the CF respiratory tract in spite of the CF patient's apparently normal array of host defenses (40, 50, 94, 100, 110). Most CF patients eventually succumb to pulmonary failure due to overwhelming infection with *P. aeruginosa* (94).

## IX. Conclusions.

Given the accumulating body of literature on pilus assembly, it was thought that it would be beneficial to investigate the effects of pilin subunit structure and of pilus-associated signal transduction on *P. aeruginosa* adherence to host cells. In this thesis, three main projects were initiated. First, the feasibility of using *lux* genes expressed from a plasmid transformed into *P. aeruginosa* for bacterial enumeration was assessed. This study was undertaken in the hope of using bacterial luminescence to enumerate bacteria bound to epithelial cells in an adherence assay. The second project involved the development of a bacterial-epithelial, whole-cell attachment assay that would allow the comparison of the adherence abilities of different strains of *P. aeruginosa* and the further characterization of the roles of pilin and pilus structure in adherence. The third project involved the characterization of two unique mutants in *pilG*, a gene required for twitching motility, bacteriophage infection, and the normal expression of pili. This study, in part, confirmed the role of the PilG protein in *P. aeruginosa* pilus assembly and contributed more detailed information about PilG's structure and function. Taken together, the work described in this thesis has helped to further our knowledge about *P. aeruginosa* pilus structure, the importance of the disulfide loop region of the pilus in adherence to epithelial cells, and the role of signal transduction in the putative pilus chemotactic system.

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# Chapter 2

## Evaluation of Bioluminescence as a Method of Enumeration for Bacteria

# A. Introduction

## I. Background Information.

Development of an *in vitro* monolayer attachment assay for *Pseudomonas aeruginosa* would require a reliable and reproducible method for detecting and enumerating both adherent and non-adherent bacteria. This led to the investigation of light production, by bacteria expressing plasmid-encoded *lux* genes, as an enumeration method. The *lux* gene was chosen as a candidate reporter system because of its ease of detection; the light emitted by the bacteria is readily and directly detected by a luminometer. It was also thought that this system would lend itself to automated detection by a photometer or image analyzer, making the assay a technically simple procedure.

The source of the cloned genes for expression of luminescence was the chromosome of the marine bacterium *Vibrio fischeri*. This organism is a symbiont in the light organs of some fish, for example *Monocentris japonicus* (6, 11, 12). The light reaction, as shown below, is catalyzed by bacterial luciferase, a 77 kDa heterodimeric enzyme made up of $\alpha$ (40 kDa) and $\beta$ (37 kDa) subunits.

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + h\nu \text{ (490 nm)} \quad (11,17)$$

Reduced flavin mononucleotide ($FMNH_2$) and a long chain fatty aldehyde (RCHO) are oxidized to flavin mononucleotide (FMN) and the corresponding long chain fatty acid (RCHOOH) with the emission of blue-green light. The naturally occurring aldehyde substrate for this reaction is thought to be tetradecanal on the basis of its identification in lipid extracts, the preference of luciferases for tetradecanal at low substrate concentrations, and the specificity of the *lux*-specific fatty acid reductase system which catalyzes the synthesis of the aldehyde substrate (11). Exogenously added decanal can be used in *in vitro* experiments requiring addition of the substrate since it diffuses freely through the bacterial membrane. The *V. fischeri* luciferase gives off light of maximum intensity when dodecanal is the substrate *in vitro* but decanal appears to cross the cell membrane more readily *in vivo* than the longer-chain aldehydes (11). The light emitting reaction begins when $FMNH_2$ bound to luciferase reacts with molecular oxygen to become a second, long-lived, enzyme-bound intermediate and, in the presence of a long chain aldehyde substrate, can be converted to a third enzyme-bound intermediate which then breaks down to produce the oxidized FMN and the long chain fatty acid at a rate dependent on the chain length of the aldehyde substrate (Figure 2.1). It is during this last part of the reaction that an excited species is produced and light is given off (17). The emission maximum of the light produced is 490 nm. With a single pulse of $FMNH_2$ added to a reaction mixture containing luciferase enzyme and aldehyde, the light intensity rises to a peak followed by an exponential decay with a half-life of 2-20 seconds at $25^oC$. The rate of decay is dependent on the source of the luciferase, the carbon chain length of the aldehyde, temperature, and pH (17).

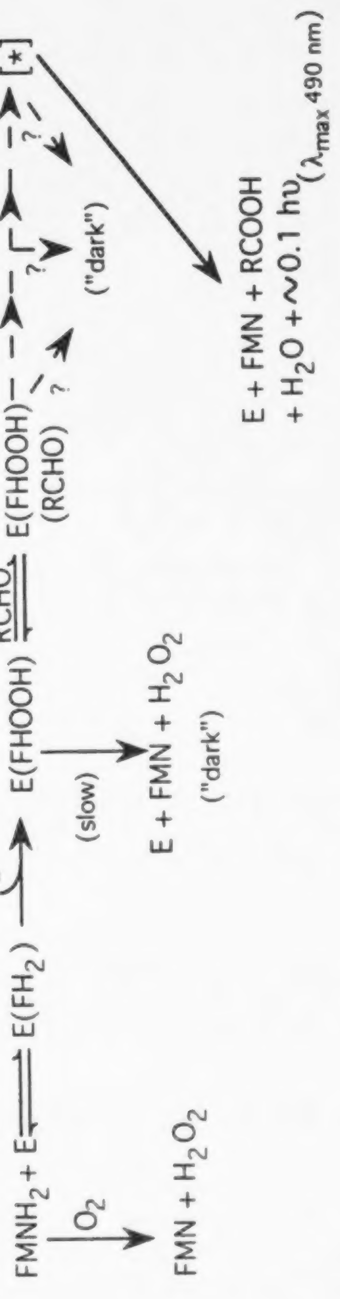
Seven *lux* genes from *V. fischeri* were required for light production in recombinant *Escherichia coli* (6). Figure 2.2 shows that these genes were located in two divergently transcribed operons and coded for gene products involved in light production and regulation of gene expression. The *luxA* and *luxB* genes code for the $\alpha$ and $\beta$ subunits of the luciferase enzyme and the *luxC, luxD*, and *luxE* gene products function in the synthesis or recycling of the aldehyde substrate. The *luxI* gene was found to code for a gene product involved in synthesis of the autoinducer protein, b-ketocaproyl homoserine lactone (6). The autoinducer (AI), which diffuses freely across the bacterial membrane, induces transcription of the luminescence genes when the extracellular level of AI reaches a critical concentration (10). The product of the *luxR* gene is required for cells to respond to the autoinducer. LuxR activates transcription of both the *luxR* and *luxICDABEG* operons after binding AI by interacting with the *lux* operator located between the two operons, and inducing transcription from both operons (5, 13, 14). This leads to a positive feedback loop since increased transcription of *luxI* results in the production of more AI, which, in turn, further activates LuxR and transcription of the two *lux* operons. The end result is the rapid and high level induction of transcription of luminescence genes at a particular cell density corresponding to the time at which AI in a growing culture reaches a critical concentration (14).

The purpose of the following experiments was to characterize the constitutive expression of *V. fischeri* lux genes expressed in *P. aeruginosa* and to determine if bacterial bioluminescence could be used to reliably and reproducibly enumerate bacteria in *in vitro* adherence assays.

## II. Choice of Bioluminescence System.

A common way to detect light production is to clone only the *luxA* and *luxB* genes for production of the luciferase enzyme on a plasmid and express that construct in the desired bacterial strain (4). *In vivo*, bacterial metabolism would provide one substrate, the

Fig. 2.1. Intermediates in the reaction of bacterial luciferase *in vitro* with reduced flavin mononucleotide ($FMNH_2$ or $FH_2$), molecular oxygen, and a long-chain aldehyde (RCHO). Steps leading to the production of an exited state [∗] and the exact mechanism for light emission are not well understood and are indicated by dashed arrows and question marks. Side reactions not resulting in light production are indicated by ("dark"). Reproduced from reference 17 with permission, © 1981 by Academic Press, Inc.


FMNH2 + E ⇌ E(FH2)
(I)
O2
FMN + H2O2
E(FHOOH)
(II)
(slow)
E + FMN + H2O2
("dark")
RCHO
E(FHOOH)
(RCHO)
(III)
?
("dark")
[*]
E + FMN + RCOOH
+ H2O + ~0.1 hυ (λmax 490 nm)

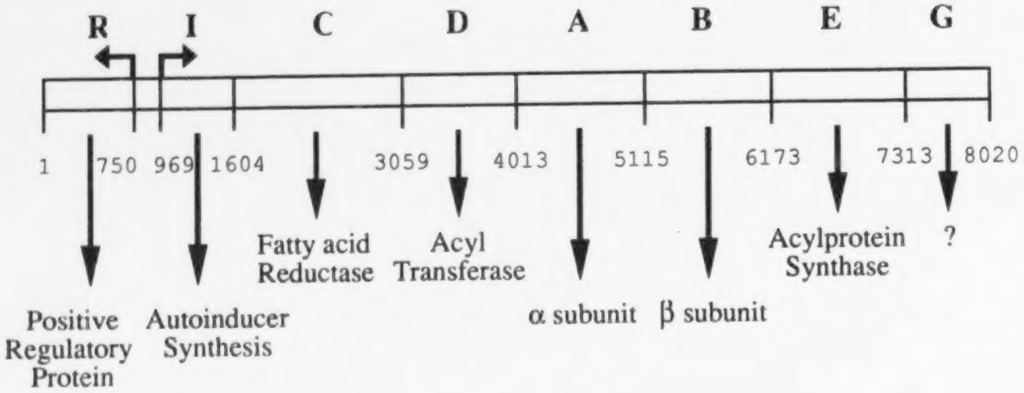

R
I
C
D
A
B
E
G
1
750
969
1604
3059
4013
5115
6173
7313
8020
Fatty acid Reductase
Acyl Transferase
Acylprotein Synthase
?
Positive Regulatory Protein
Autoinducer Synthesis
α subunit
β subunit

Fig. 2.2. *lux* gene organization for *V. fischeri*. Bold letters indicate the individual *lux* genes, small arrows indicate the direction of transcription of the operon, and large arrows indicate the functions of the gene products. Numbering of bases starts from the last codon of the *luxR* gene and extends to the last codon of the *luxG* gene, with the intermediate numbers indicating the first base of the methionine start codon for each gene (3, 16).

$FMNH_2$, and the other substrate, the fatty aldehyde in the form of decanal, would be supplied exogenously as a vapour to stimulate light production. *In vitro*, light production by isolated luciferase enzyme was initiated by the addition of $FMNH_2$ into a solution containing luciferase and decanal (decanal was supplied as a sonicated suspension in water at 0.01% (v/v)) and followed immediately by detection of the light produced due to the "pulse" of $FMNH_2$ (2, 8). A second method, developed by Boylan, Pelletier and Meighen to facilitate expression of luciferase in eukaryotes, involved the fusion of the *luxA* and *luxB* genes by abolishing both the stop codon of *luxA* and the initiation codon of *luxB* and changing the intergenic region to a 10-amino acid linker peptide between the two genes in order to create one translational unit for the heterodimeric luciferase enzyme. The expression of this monocistronic *luxAB* gene in *E. coli* resulted in a level of light emission comparable to that for *E. coli* containing the unfused *lux* genes (30-75% depending on mutations made to the *luxA*-*luxB* intergenic region) (4).

Another method for light detection was developed by Shaw and Kado by removing the *luxI* and *luxR* regulatory genes and cloning the other *lux* genes from *V. fischeri* behind a promoter to allow constitutive bioluminescence in a culture (15). The presence of the genes required for production of the aldehyde substrate eliminated the need for exogenously added decanal. This was the primary reason we chose to use this system for detection of bacteria in epithelial cell-binding experiments. Bacteria in liquid culture could simply be diluted in an appropriate buffer, pipetted into cuvettes, and the light produced read directly by a luminometer. The minimal processing of the bacteria required to obtain bioluminescence readings appeared to make this system easy to adapt to an adherence assay protocol.

## B. Materials and Methods

### I. Strains, Culture Conditions and Plasmids.

The strains and plasmids used in these studies are listed in Table 2.1. Note that strains or plasmids in parentheses are included for discussion purposes only and were not used in experiments. *P. aeruginosa* strains were maintained on Luria-Bertani medium agar plates (LBA). Plates were supplemented as needed with tetracycline (100 µg/ml) for *P. aeruginosa* strains containing pDLUX-1.

Table 2.1. Bacterial strains and plasmids used in this study.

| | Genotype or description[b] | Source or reference |
|---|---|---|
| **Strains** | | |
| (*Escherichia coli* MM294)[a] | *supE44 hsdR endA1 thi* | (1) |
| *Pseudomonas aeruginosa* PAO1 | | (9) |
| *Pseudomonas aeruginosa* BLP3 | Tn*501 pilA* mutant of PAO1 | B. L. Pasloske |
| **Plasmids** | | |
| pDLUX-1 | Tc$^{r}$ | D. E. Zoutman |
| (pLAFR1) | Tc$^{r}$ | (7) |
| (pUCD607) | Ap$^{r}$, Km$^{r}$, Sp$^{r}$ | (15) |

[a] Parenthesis indicate strains or plasmids included only for the purpose of discussion.

[b] Abbreviations: Ap, ampicillin; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline; r, resistance.

## II. Bacterial Luminescence Assay.

For quantitation of luminescence, a single isolated colony was used to inoculate 1 ml of Luria-Bertani medium (LB), pH 7.2, containing 50 µg/ml tetracycline and this culture was grown approximately 5-6 hours at 37$^{o}$C, 50 rpm. 0.1 ml of this was used to inoculate 10 ml of LB with tetracycline and this culture grown overnight at room temperature, 125 rpm to a Klett meter reading of 75. Under these conditions, the cultures had previously been shown to be in the mid-log phase of growth at the time of the assay (J. Boelens, personal communication). The culture was diluted in five-fold series in phosphate-buffered saline (PBS), pH 7.2, to measure light output for 1 ml aliquots of each dilution. Each dilution was also further diluted to appropriate concentrations and plated out on LBA with 100µg/ml tetracycline to determine the concentration of bacteria in each tube. Agar plates for the plate counts were incubated at 30$^{o}$C for 48 hours or at 37$^{o}$C for 24 hours, colonies counted as the plates equilibrated to room temperature, and light output confirmed by observing the colonies in a darkened room.

Light output was measured using a Wallac LKB 1251 luminometer. Aliquots (1 ml) of solutions to be read were placed in 51 mm x 12 mm polystyrene cuvettes, loaded in the luminometer and the light emitted was quantitated over a 5 or 10 second interval at 25$^{o}$C . The luminometer was set for continuous mixing of the cuvette and integration of the light detected for the set time interval. All assays were read against a blank of 1 ml of PBS, pH 7.2 or PBS, pH 7.2 with 3% (v/v) LB as appropriate. Luminescence was expressed as mV/sec/ml. Graphing was done with the Cricket Graph v1.3.2 program designed for the Macintosh Computer.

Assays at 30°C or 37°C were carried out essentially as above. Bacterial cultures were grown as noted but at 30°C or 37°C as appropriate and the incubation times shortened to make sure the cultures were in mid-log phase at the time of the assay. In each case, dilutions and light readings were done at the appropriate temperature in order to maintain the bacteria at that temperature for the entire assay.

### III. Bioluminescence Temperature Variability Assay.

Bacteria were grown as for a 37°C luminescence assay, diluted with PBS, pH 7.2 at 37°C, and the luminescence of a $10^{-3}$ dilution read for 5 seconds at 25°C in the luminometer. This was repeated every 5 minutes for 20 minutes then at 15 minute time intervals after 30 minutes. At each time interval, the temperature of the culture was determined using a flame-sterilized thermometer. Aliquots were removed for plate counts at the beginning of the assay and every 30 minutes thereafter.

### IV. Room Temperature Bioluminescence Stability Assays.

The bacterial inoculum was grown as for the bacterial bioluminescence assay. 0.2 ml of the first culture was used to inoculate 20 ml of LB with tetracycline and this culture grown overnight at room temperature, 125 rpm to a Klett meter reading of 75. The culture was diluted to $10^{-3}$ in phosphate-buffered saline (PBS), pH 7.2 and plated out on LBA with 100µg/ml tetracycline. Aliquots of 1 ml (in triplicate) were assessed for light output. Plates for plate counts were incubated at 37°C overnight and counted as noted in the bacterial bioluminescence assay. At 30 minute time intervals, the cuvettes were vortexed and read again in the luminometer and aliquots from the plateout dilutions were plated out as before. Between readings, all tubes remained standing at room temperature.

The stability of luminescence over time was also determined with the addition of LB to the PBS used for dilutions. The assay was performed as above except that the PBS used for diluting the culture for luminometer readings and plateouts had 20% (v/v), 10% (v/v), 5% (v/v), 3% (v/v), 2% (v/v), or 1% (v/v) LB added to it.

## C. Results

### I. Determination of a Linear Relationship Between Light Output and Bacterial Concentration.

Prior to the work on this thesis, the *luxCDEAB* gene cassette from pUCD607 (15) had been cloned as a 16 kilobase (kb) fragment into the EcoRI site of the low copy number vector pLAFR1 (7) to produce a 37.6 kb plasmid called pDLUX-1. pDLUX-1 was transformed into a number of strains and mutants of *Pseudomonas aeruginosa* by triparental mating using the *E. coli* helper-strain MM294/pRK2013, to provide the light-producing strains used for all bioluminescence experiments.

It had been observed that there seemed to be a linear relationship between the amount of light detected from a liquid culture of bacteria containing the pDLUX-1 plasmid and the concentration of bacteria in the culture. Before serious consideration as an enumeration method, light output from bacterial cultures needed to be shown to be linear with respect to bacterial concentration. PAO1/pDLUX-1 and BLP-3/pDLUX-1 were each grown from a single colony in LB containing 50 µg/ml tetracycline by incubating them at room temperature, 125 rpm, to mid-log phase (approximately 2 x $10^8$ colony forming units/ml (CFU/ ml)). They were then diluted in a five-fold dilution series in PBS, pH 7.2, their light output assessed, and plated out to determine CFU/ml. Figure 2.3 shows that a linear relationship exists between the light output from the cultures and the concentration of bacteria. This indicated that the bacterial bioluminescence system had potential to be a useful method for enumerating bacteria. An additional benefit was that only viable bacteria would be detected since non-living cells would not be able to carry out the light-producing reaction.

## II. Variability of Bioluminescence With Temperature.

The linear relationship between bacterial number and bioluminescence was demonstrated at room temperature but future adherence assays would be carried out at 37°C, so it was necessary to determine if the relationship held at higher temperatures. An experiment similar to the previous one was carried out with PAO1/pDLUX-1 and BLP-3/pDLUX-1 at 30°C and 37°C. Bacterial concentration and bioluminescence were again shown to have a linear relationship but the amount of light produced by the cultures decreased as the growth temperature increased. This can be seen by the decreasing slopes of the lines in figure 2.4. There are a number of possible explanations for this decrease in bioluminescence including instability of one of the enzymes in the aldehyde substrate biosynthetic pathway, the luciferase enzyme, or the mRNA transcripts of *lux* gene products at higher temperatures but the only instability reported in the literature is that of the luciferase enzyme itself (11). The decrease in light activity could also be due to increased yield in one or more of the side ( or "dark") pathways in the bacterial bioluminescence reaction (Figure 2.1). The results of this experiment indicated that binding assays would have to be performed at room temperature in order to maximize the detection of bacteria by light output since at higher temperatures,

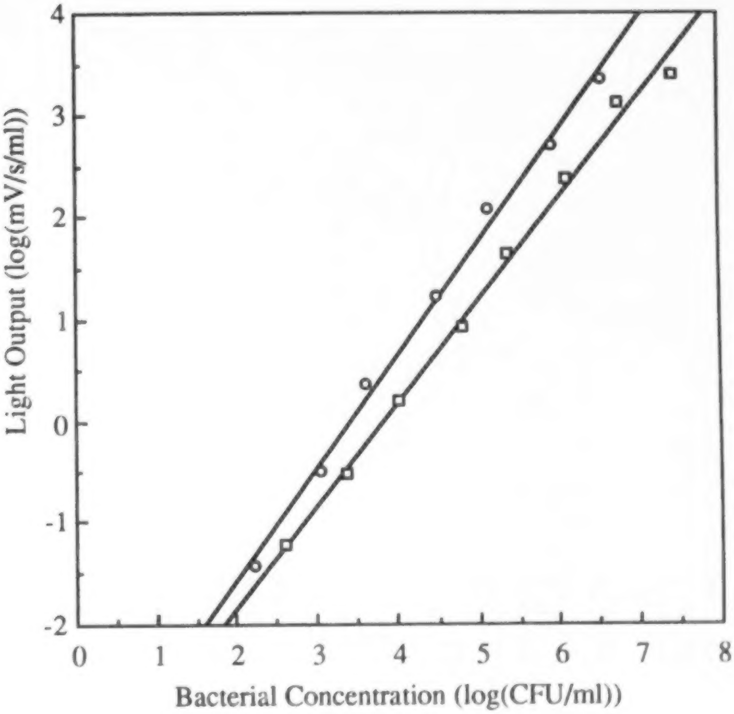
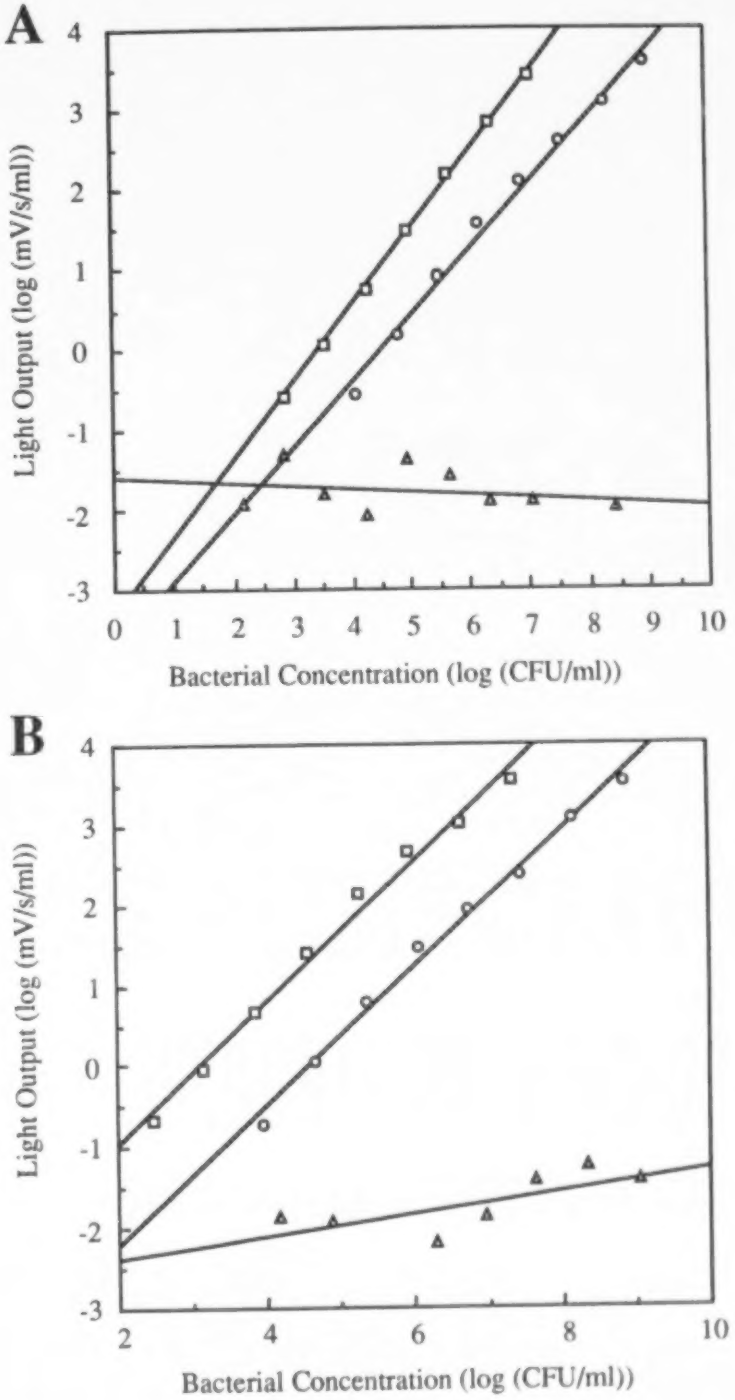


Fig. 2.3. Relationship between bioluminescence and concentration of bacteria for PAO1/pDLUX-1 (□, $r^2$=0.995) and BLP3/pDLUX-1 (O, $r^2$=0.994). Bacteria were grown at room temperature, with shaking, to mid-log phase then diluted in a five-fold dilution series in PBS, pH 7.2. Plate counts were done for each dilution and light output was determined by reading 1.0 ml of each dilution in a luminometer.

Figure 2.4. Temperature dependence of bacterial bioluminescence. Light output from *P. aeruginosa* PAO1/pDLUX-1 (**A**) and BLP3/pDLUX-1 (**B**) was observed to decrease with increasing growth temperature, resulting in decreasing sensitivity of detection of bacterial concentration. Growth temperatures assayed were 23°C (□), 30°C (O), and 37°C (Δ). Correlation coefficients for PAO1/pDLUX-1: $r^2$=0.999, 23°C; $r^2$=0.993, 30°C; $r^2$=0.090, 37°C. Correlation coefficients for BLP3/pDLUX-1: $r^2$=0.991, 23°C; $r^2$=0.992, 30°C; $r^2$=0.512, 37°C.


A
Light Output (log (mV/s/ml))
4
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
6
7
8
9
10
Bacterial Concentration (log (CFU/ml))
B
Light Output (log (mV/s/ml))
4
3
2
1
0
-1
-2
-3
2
4
6
8
10
Bacterial Concentration (log (CFU/ml))

the sensitivity decreased.

Confirmation of the instability of the light output was obtained when light producing bacteria were subject to changes in temperature. PAO1/pDLUX-1 and BLP-3/pDLUX-1 were grown at 37°C, diluted with PBS at 37°C, then the light output and temperature of the culture determined at various time intervals as the culture was allowed to cool to room temperature. Fig. 2.5 shows that light production by PAO1/pDLUX-1 continued to increase up to approximately 30 minutes after the culture's temperature had dropped to 22°C, at which time light output was stable for 40-50 minutes then began to decrease. Similar results were obtained for BLP-3/pDLUX-1 (data not shown). These data suggest that *in vivo*, light output is very sensitive to fluctuations in temperature. The maximum light output observed occurred at room temperature and decreased with increasing temperature.

### III. Stability of Light Output at Constant Temperature.

The next question was whether or not the light output from a bacterial culture was stable over time when held at a constant temperature. This would be necessary in a binding assay as bacteria would be incubated on epithelial monolayers for up to 1-2 hours for attachment. It would be important for the light output to be stable over that time period so that the light output could be related to a standard curve to determine numbers of adherent bacteria.

PAO1/pDLUX-1 and BLP-3/pDLUX-1 were grown at room temperature, diluted in PBS, and their light output monitored for approximately 2 hours at 25°C. Both cultures showed a sharp decrease in bioluminescence after 10 minutes. This continued such that the light output dropped by 30-50% after 45 minutes and 82-86% after 2 hours (Fig. 2.6). This could have been due to a decrease in energy available for luminescence in the cells since they were in PBS, which does not contain an energy source. The experiment was repeated with the addition of 20% , 10%, 5%, 3%, 2%, or 1% LB to the PBS in order to provide enough energy to stabilize the light output but not promote growth of the culture. The greatest stability in light output and bacterial concentration was obtained when PBS+3% LB was used as the incubation buffer, as shown in Fig. 2.6. This indicated that the instability of the light output over time was due to the depletion of energy in the bacteria and that the addition of 3% LB to the PBS would stabilize the light ouput enough to allow enumeration of bacteria with this method.

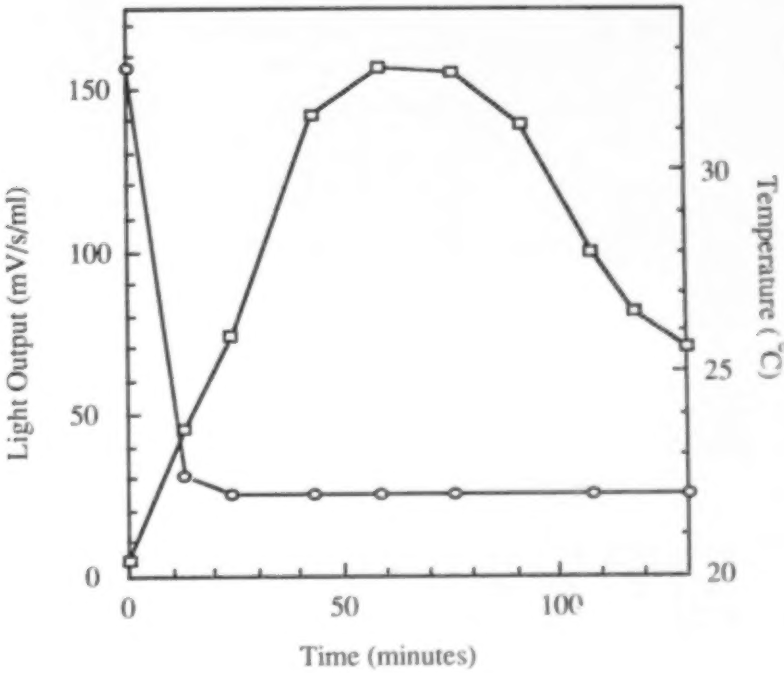
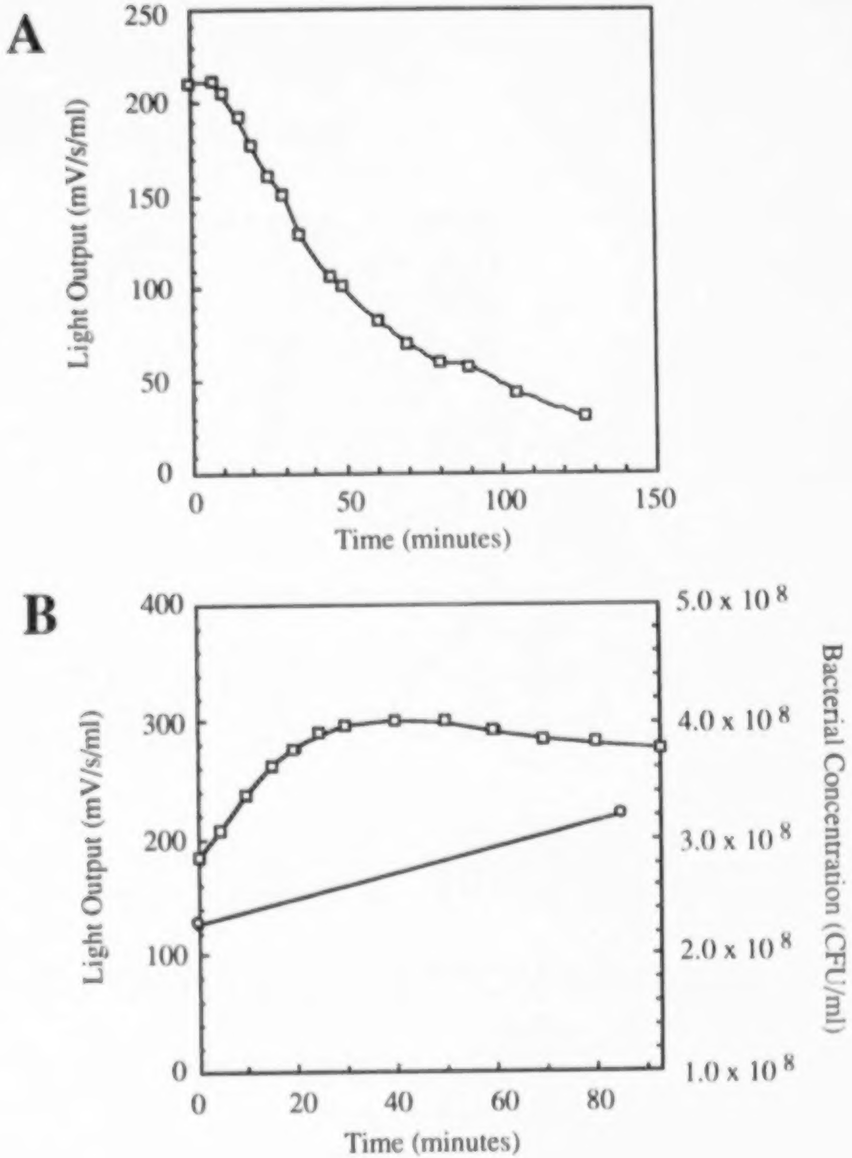


Fig. 2.5. Variability in light output with temperature change. PAO/pDLUX-1 was grown to mid-log phase at 37°C, then diluted in PBS, pH 7.2 and its light output (□) determined at various time intervals by reading in a luminometer. Temperature of the culture as it cooled (O) was also determined for a total of approximately 2 hours.

Fig. 2.6. Stability of PAO1/pDLUX-1 bioluminescence with time. The light output from a mid-log phase culture of PAO1/pDLUX-1 grown at room temperature and diluted in PBS, pH 7.2, was measured in a luminometer at various time intervals for approximately 2 hours. (**A**) A similar culture was also diluted in PBS + 3% (v/v) LB, pH 7.2, then its light ouput determined for just over 90 minutes (□) and its beginning and ending bacterial concentration determined by plate count (O). (**B**)


A
Light Output (mV/s/ml)
250
200
150
100
50
0
0
50
100
150
Time (minutes)
B
Light Output (mV/s/ml)
400
300
200
100
0
0
20
40
60
80
Time (minutes)
Bacterial Concentration (CFU/ml)
5.0 x 10 8
4.0 x 10 8
3.0 x 10 8
2.0 x 10 8
1.0 x 10 8

## D. Conclusions

In order to develop a whole cell adherence assay to determine the ability of bacteria to bind to eukaryotic epithelial cells, it was first necessary to devise an appropriate method for detection of the bacterial cells. The detection method needed to be reliable, reproducible and relatively simple to perform. It had previously been found that *P. aeruginosa* transformed with a plasmid containing luminescence genes from *V. fischeri* on a low copy number plasmid seemed to maintain a linear relationship between light output and bacterial concentration. This relationship could be exploited as an enumeration method providing it was accurate and reproducible under the conditions required for the adherence assay. Characterization of this relationship was undertaken in order to determine if it would be suitable for routine use in a bacterial whole cell adherence assay.

The *V. fischeri lux* system was chosen because of its ease of detection; solutions containing light-producing bacteria could be easily detected and enumerated in a luminometer. Bacteria bound to epithelial cells could also be enumerated using the luminometer by seeding the eukaryotic monolayers onto plastic slides in Leighton tubes for growth of the epithelial cells and, after the adherence assay, placing the entire slide into a cuvette filled with buffer for detection of light emitted. It was hoped to take advantage of this ease of counting by developing an assay using automated detection with a microtitre plate reader or photon image analyser.

The inclusion of all the genes necessary for light production on the cloned *lux* cassette was another reason for choosing the *V. fischeri lux* system. These genes were under control of the tetracycline resistance gene promoter from the plasmid pBR322 and they included all of the genes necessary for light production in *E. coli* or *P. aeruginosa* except for the regulatory genes normally present in *V. fischeri*. This meant that a bacterial host containing a plasmid with the *lux* cassette produced light constitutively without a requirement for any exogenously added substrate. For the adherence assay, this allowed a minimum of manipulation of the bacteria bound to epithelial cell monolayers in order to obtain luminescence readings from the bacteria, making the detection steps very simple to perform.

Characterization of the light output from *P. aeruginosa* containing the *lux* cassette on a plasmid (pDLUX-1) indicated that a linear relationship existed between the light output by the bacteria and the concentration of bacteria in the solution tested. The fact that this relationship existed made it possible to investigate the usefulness of this method of detection. Obviously, the linear relationship between bioluminescence and cell number made it possible to accurately and reproducibly relate the measured bioluminescence to numbers of bacteria in a solution or to bacteria bound to a monolayer on a slide. The light output from the bacteria was stabilized over time by adding 3% (v/v) LB medium to the PBS in which the dilutions and measurements were done, enabling the light output to be more consistent for at least two hours. This would enable the bacteria to be measured over the course of an adherence assay and to have consistent light output during the assay, but not increase significantly in bacterial number. Another benefit of this system of detection was that only viable bacteria were detected as only viable bacteria were able to bioluminesce.

Unfortunately, as suitable as the bioluminescence system appeared to be, there were problems with using it as a detection method. In *P. aeruginosa* the *V. fischeri lux* system showed decreased bioluminescence at temperatures above room temperature. The epithelial cell adherence assay would require incubations at $37^{o}$C for periods on the order of an hour and the low bioluminescence at $37^{o}$C would not allow very sensitive detection of the bacteria. The light output at this temperature is unstable as the luciferase enzyme itself was found to be unstable at $37^{o}$C (11).

A second problem was encountered when *P. aeruginosa* expressing *lux* genes was subject to temperature changes. When a suspension of bacteria was allowed to cool to room temperature from $37^{o}$C, the light output was unstable for a prolonged period of time. This precludes the possibility of carrying out an adherence assay at $37^{o}$C and subsequently carrying out measurements of adherent and non-adherent bacteria at room temperature since a delay of at least thirty minutes would be required before assaying the light output. In this period of time, statistically significant bacterial multiplication could take place or the nature of the interaction between the bacteria and the epithelial cells could change, making the relationship between measured light output and the true nature of the bacterial-epithelial cell interaction uncertain.

A minor problem not previously mentioned was technical in nature. This would not have prevented use of the adherence assay but it would have prevented easy automation. In order to be able read emitted light from a microtitre plate in the equipment we had available, we would have had to use special black plastic microtitre plates that would prevent light from adjacent wells contaminating the well being detected. These were not available as sterile plates, nor were they available with coatings for eukaryotic cell culture. While this problem may not have been insurmountable, it was yet another concern in the use of this method.

Because of the various problems discussed above, most notably the instability of the light output with higher temperatures or with temperature changes, it was decided that for our purposes the *V. fischeri lux* system would be unsuitable as a method of enumerating bacteria in an adherence assay. There are other bacterial luminescence systems that could also have been possible candidates for the enumeration method but it was not until close to the completion of this work they were becoming better characterized. With the information now available, the *lux* genes from *Xenorhabdus luminescens* would likely be the best choice for this assay since the thermal stability of its luciferase is higher ($t_{1/2} > 3$ hours at 45°C) than the stability of either the *V. harveyi* luciferase ($t_{1/2} = 5$ minutes at 45°C) or the *V. fischeri* luciferase (11). The *lux* system was replaced with a direct plate count method which had the advantage of including only living bacteria in the enumeration. This method and the development of the adherence assay is described in detail in Chapter 3.

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# Chapter 3

## Studies on *Pseudomonas aeruginosa* Adherence to Cultured Human Lung Pneumocytes[1].

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[1] A version of this chapter has been published. Farinha, M.A., B.D. Conway, L.M.G. Glasier, N.W. Ellert, R.T. Irvin, R. Sherburne and W. Paranchych. 1994. Infect Immun. **62**:4118-4123.

## A. Introduction

### I. Background Information.

As mentioned in Chapter 2, research into the feasibility of a *Pseudomonas aeruginosa* vaccine and studies on the pili of *P. aeruginosa* led to the requirement for an *in vitro* whole-cell binding assay that would allow the comparison of different strains of this organism. Because of the difficulties associated with using a bioluminescent method of bacterial enumeration, it was decided to use a method whereby bacteria bound to eukaryotic monolayers could be recovered and enumerated by a plate count method. To devise a method that would be simple and reproducible, a number of parameters needed to be investigated. Which epithelial cell line was to be used and at what density, bacterial growth conditions, adherence time, washing conditions to remove unbound bacteria, and calculation of binding constants were among the parameters that were standardized for the adherence assay. This assay was then used to compare the adherence characteristics of different *P. aeruginosa* strains.

### II. Ligand-Receptor Theory and Calculations.

The mathematical model chosen to account for bacterial (ligand) binding to a monolayer of cells (receptors) was the Clark's Occupancy Theory (1) since the assumptions of this model fit the observed adherence conditions between the bacteria and monolayers. Those assumptions are:

1. Interaction between ligand (L) and receptor (R) is *reversible*: the association is a *bimolecular* process and the dissociation is a *monomolecular* one (1).

While it was recognized that bacteria may express more than one adhesin and that more than one may be involved in bacterial binding, it was also recognized that for the purposes of this assay, it was sufficient to consider the cumulative effect of these potential adhesins which was the binding of the bacteria to a cell in the monolayer.

2. All receptors are *equivalent* (they have the same affinity for the ligand) and *independent* (the affinity remains unchanged when the receptors become progressively saturated); the occupancy of a receptor does not modify the affinity for the ligand of the remaining free receptors (1).

The whole cell binding assay characterizes the overall binding of a complex ligand (the bacterium) to a complex receptor (the eukaryotic cell) by assuming that the interaction of one or more than one potential ligand on the same bacterium with one or more than one potential receptor on the same eukaryotic cell results in the binding of one bacterium with one eukaryotic cell.

3. The biological response is *proportional* to the number of occupied receptors: a linear relationship exists between binding and effect (1).

The effect is internalization of the bacteria which has been shown for *P. aeruginosa* with the A549 human lung pneumocyte cell line used (7). A linear relationship between binding and internalization has been neither proved nor disproved since it was not investigated. Also, the rate at which internalization occurs has not been determined. It was assumed that this rate would be low enough that the number of internalized bacteria would be negligable compared to the number of adherent bacteria, in support of assumptions 1 and 5.

4. The response is measured when the interaction between ligand and receptor has reached a state of *equilibrium* (1).

The specific nature of the equilibrium state of bacteria binding to A549 cell monolayers was not addressed in detail in these studies. Instead, binding was always assessed at the same time interval after addition of the bacteria to the monolayers so that approximately the same degree of equilibrium would have been reached by the different systems.

5. The ligand can exist in only two states, free or receptor-bound; in particular, it is assumed that it is not degraded or bound to other acceptor sites. Furthermore only a negligible fraction of the ligand is bound to the receptor: the concentration of free ligand is thus approximately equal to the total concentration of ligand (1).

In the adherence assay, bacteria are either free or receptor-bound, they exist in only two states. It was shown that the concentration of free bacteria was not significantly different at the beginning and end of the assay, indicating that there was no appreciable change to the ligands over the course of the assay. Also, it was observed that at most, the concentration of bound bacteria was less than one one-thousandth that of the unbound bacteria, satisfying the condition that the concentration of free ligand be equal to the total concentration of ligand.

Since no distinction was made between bound bacteria and internalized bacteria, the equation describing the interaction of ligand (L) and receptor (R) was simplified to:

$$R + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} RL$$

where the rate constants of the forward and reverse reactions ($k_a$ and $k_d$, respectively) characterize the equation. A given system characterizing the interaction of a bacterial culture with A549 pneumocytes was described by both a direct plot of bound bacteria vs. free bacteria (recall that one assumption is that unbound L = free L in this model) and a Scatchard plot of bound/ free bacteria vs. bound bacteria. The Scatchard is a useful plot for characterizing binding since for Clark's model, it is a straight line and can be used to calculate the equilibrium dissociation constant, $K_D$ (1). All calculations and plots were done as described in section 3.B.VIII.

## B. Materials and Methods

### I. Bacterial Strains and Culture Conditions.

The bacterial strains used in these studies are listed in Table 3.1. Bacteria were maintained on tryptic soy broth (TSB) plates made by adding agar to 1.5% (w/v) to tryptic soy broth, pH 7.0. While in use, bacteria were subcultured approximately once a month and stored at 4°C on TSB agar. Long-term storage of strains was accomplished by either lyophilizing plate cultures in 24% (w/v) skim milk and storing under vacuum at room temperature or by storing a liquid culture in 10% (v/v) glycerol at -70°C (12).

### II. Eukaryotic Cell Culture Conditions.

The human lung carcinoma A549 cell line (Table 3.1) was obtained from the American Type Culture Collection (Rockville, Maryland). These cells were grown in 25 cm$^2$ tissue culture flasks (Miles Laboratories, Inc., Naperville, IL) in Gibco Waymouth MB 752/1 medium (cat. # 51400-026) supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 56°C for 30 minutes), 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotic solution (100 U/ml penicillin, 100 mg/ml streptomycin). Cells were routinely incubated at 37°C in 7.5% $CO_2$ in a humidified incubator and were passaged at 10-day intervals. The cell monolayer was detached by a 5-7 minute incubation in Trypsin-EDTA solution (0.5 mg/ml trypsin, 0.53 mM disodium EDTA, 0.85 mg/ml NaCl in CMF-PBS, pH 7.2 (140 mM NaCl, 4 mM KCl, 0.5 mM $Na_2HPO_4$, 0.1 mM $KH_2PO_4$, 11 mM glucose)) at 37°C. Long-term storage of cells was under liquid nitrogen in media with 5% (v/v) dimethylsulfoxide (DMSO). Passages 84-90 were used in these experiments.

Table 3.1. Bacterial strains and cell cultures used in this study.

| Strain or cell line | Genotype or description[a] | Source or reference |
|---|---|---|
| *Pseudomonas aeruginosa* | | |
| PAK | | (30) |
| PAK-N1 | $Tc^r$ insertion into *rpoN* in PAK | (15) |
| PAK-NP | $Tc^r$ insertion into *pilA* in PAK | (17) |
| PAO1 | Prototroph, $FP^-$ | (13) |
| PAO-MP | $Cm^r$ insertion into *pilA* in PAO1 | (10) |
| BLP3 | Tn*501 pilA* mutant in PAO1 | B. L. Pasloske |
| Cell Culture | | |
| A549 | Human lung carcinoma<br>ATCC: CCL 185, Batch# F-8669 | (19) |
| CCRF S-180 II | Mouse sarcoma 180<br>ATCC: CCL 8 | (11) |
| HEL 299 | Human embryonic lung<br>ATCC: CCL 137 | (22) |

[a] $Cm^r$, chloramphenicol resistance; $Tc^r$, tetracycline resistance.

The human embryonic lung HEL cell line (Table 3.1) was also obtained from the American Type Culture Collection (Rockville, Maryland), and the cells were grown in 25 cm$^2$ tissue culture flasks (Miles Laboratories, Inc., Naperville, IL) in Gibco Minimum Essential Medium (MEM, cat. # 11700-010) supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 56°C for 30 minutes), 2 mM L-glutamine, and 25 µg/ml gentamicin sulfate. Cells were routinely incubated at 37°C in 7.5% $CO_2$ in a humidified incubator and were passaged at 7-day intervals. The cell monolayer was detached by a 10-15 minute incubation in Trypsin-EDTA solution at 37°C. Long-term storage of cells was under liquid nitrogen in media with 5% (v/v) dimethylsulfoxide (DMSO). Cells were used for approximately 30 transfers only.

The mouse sarcoma CCRF S-180 II cell line (Table 3.1) was a kind gift from Dr. W. Gallin (Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9). Cells were grown in 25 cm$^2$ tissue culture flasks (Miles Laboratories, Inc., Naperville, IL) in Gibco Dulbecco's Modified Eagle Medium (D-MEM, cat. # 12100-046) supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 56°C for 30 minutes), 1 mM sodium pyruvate, 2 mM L-glutamine, and 25 µg/ml gentamicin sulfate. Cells were routinely incubated at 37°C in 7.5% $CO_2$ in a humidified incubator and were passaged at 7 to 10-day intervals. The cell monolayer was detached by a 5-7 minute incubation in Trypsin-EDTA solution at 37°C. Long-term storage of cells was under liquid nitrogen in media with 5% (v/v) dimethylsulfoxide (DMSO).

### III. Epithelial Cell Adherence Assay.

*P. aeruginosa* cultures were maintained on TSB plates with antibiotics as required. TSB (100 ml) supplemented with 5 mM $MgCl_2$ (with antibiotics as required) was inoculated at 1% (v/v) with liquid culture grown from a single colony for 6-8 hours at 37°C, and grown statically in a 500 ml culture flask at 37°C for 16 hr. The bacteria were washed once with Hank's balanced salt solution (HBSS) with supplements (1 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.3) (7), adjusted to a concentration of approximately 2 x $10^9$ CFU/ml and diluted in a combination of two-fold and four-fold series. The ratios of bacterial cells:epithelial cells covered a range of 2000:1 to 15:1 in order to generate the adherence profiles. A small amount of the washed bacteria was saved for a bacteriophage (phage) sensitivity assay, described in section 3.B.IV.

Adherence assays were performed using 1 x $10^5$ A549 cells (in Waymouth's medium without antibiotics) seeded per well into 24 well tissue culture dishes (Corning) and incubated overnight. Prior to the assay, monolayers were washed three times with pre-warmed HBSS with supplements and monolayers from three wells were trypsinized (with 0.3 ml per well of Trypsin-EDTA solution), counted in a hemacytometer, and averaged to determine the density of the A549 monolayers. Dilutions of bacterial suspensions (0.3 ml aliquots) were overlaid onto the A549 cell monolayers in triplicate and incubated for one hr at 37°C with gentle mixing (70 rpm). The remaining volume of the most concentrated inoculum was also incubated under the assay conditions and plated out at the end of the hour incubation to provide a control for bacterial multiplication during the assay. A second control was provided for recovery of the bacteria by trypsinization; 0.3 ml of the sixth inoculum (chosen because its bacterial concentration was on the order of concentrations of A549-bound bacteria recovered) was mixed with an equal volume of the Trypsin-EDTA solution in a polystyrene tube that had been blocked overnight with Waymouth's medium and washed with HBSS with supplements in the same manner as the monolayers. The bacteria were incubated with the trypsin for 5 minutes at 37°C, 70 rpm, then 0.3 ml of Trypsin Inhibitor solution (0.5 mg/ ml soybean trypsin inhibitor in $Ca^{++}$, $Mg^{++}$-free PBS, pH 7.2) was added and the trypsin control diluted in PBS, pH 7.2 and plated on appropriate media.

Non-adherent bacteria were removed after the incubation period by four successive washes with HBSS with supplements (0.5 ml) for 30 seconds at 37°C, 70 rpm. Washes were pooled, diluted in PBS, pH 7.2 and plated out on TSB plates (with antibiotics as required). Bacteria bound to the A549 cells were then recovered by trypsinization of the monolayers. Trypsin-EDTA solution was added to monolayers with bound bacteria (0.3 ml per well), incubated at 37°C, 70 rpm for 5 minutes, the reaction stopped by addition of an equal volume of Trypsin Inhibitor solution and the cells recovered by vigorous pipetting. Bound bacteria were counted by plating 10-fold serial dilutions (in PBS, pH 7.2) onto TSB agar plates (with antibiotics as required). All plates were incubated at 37°C overnight and colony counts were carried out with plates containing 30 to 300 colonies. Technical assistance for the adherence assays was provided by Neil Ellert, Linda Glasier, Stasia Paczkowski and Kathleen Volpel.

## IV. Bacteriophage Sensitivity Assays.

Pf1 (5, 26), a pilus tip-binding filamentous phage and the pilus side-binding phages PO4 (3, 4) and F116 (20, 25), were obtained from D.E. Bradley (Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada, A1C 5S7). B9, a pilus side-binding phage with a noncontractile tail was obtained from R.E.W. Hancock (Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1W5). Phage sensitivities were assayed by the following method: a drop of overnight bacterial culture was added to molten 0.3% TSB agar held at 50°C, mixed briefly, then poured onto a pre-warmed (37°C) TSB agar plate. The agar was allowed to solidify, then 5 µl aliquots of high titer Pf1, PO4, F116, and B9 phages were spotted onto different sectors of the plate and the plate was inverted and incubated at 37°C for approximately 6-10 hours. Sensitivity was noted as clear areas in the bacterial lawn around the spotted phages.

## V. Negative Stain Preparations and Electron Microscopy.

Cells for electron microscopy were absorbed onto 3 mm copper grids coated with 0.25% formvar in ethylene dichloride. Bacterial cells were then negatively stained with 1% (w/v) phosphotungstic acid (pH 7.0). The grids were examined using a Philips model 410 transmission electron microscope using an accelerating potential of 80 kV. Images were recorded with Kodak 4489 electron microscope film. All electron microscopy was carried out by Richard Sherburne (Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7).

### VI. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting.

Whole protein cell lysates were prepared and analyzed on 12.5% SDS-PA gels as described in section 4.B.VIII. Gels were subsequently stained with Coomassie Brilliant Blue R-250 or transferred onto nitrocellulose membranes (see section 4.B.IX) by the method of Towbin *et al* (28). Membranes were incubated with preadsorbed anti-PAO pili-specific antiserum (section 4.B.IX) and bound antibody was detected with $^{125}$I-labeled protein A.

### VII. LD$_{50}$ Assays.

The A.BY/SnJ mice used in this study are a congenic strain developed by Jackson Laboratory, Bar Harbor, Maine (21). Both male and female mice, approximately eight weeks of age and weighing 20 ± 2 grams, were obtained from an internal breeding colony maintained by our laboratory and housed at the University of Alberta's Biosciences Animal Services. Routine serology testing was performed on these animals to insure they were free of infectious agents. The mice were group housed in filter topped cages and fed standard mouse diet and water *ad libitum*. All mice were cared for under Canadian Council on Animal Care guidelines and approved by the University of Alberta's animal care committee.

To determine LD$_{50}$ values for *P. aeruginosa* strains, eight week old mice (20 g, both sexes, 8 or 10 per assay) were given intraperitoneal injections with varying numbers of wild-type or mutant *P. aeruginosa* cells. Following challenge the mice were monitored on a hourly basis between 16 and 48 hours. All surviving mice were euthanized after the 48 hours. LD$_{50}$ values were determined by plotting survivors (at 48 h) vs. bacterial concentration used and estimating the bacterial concentration yielding a 50% survival. All animal studies were carried out by Neil Ellert and Linda Glasier.

### VIII. Calculations and Data Analysis.

Two graphical representations were used to characterize bacterial adherence to eukaryotic monolayers. A direct binding curve (bound bacteria vs. unbound bacteria) detailed the dose dependent binding between the ligand (bacteria) and the receptor (eukaryotic cell), indicating that the more ligand added to the system, the higher the level of binding until all the receptors became filled and the system was saturated. The dissociation constant ($K_D$) and total number of receptors ($R_T$) that characterized the system were computed from the best-fit curve of a Scatchard plot (bound bacteria/unbound bacteria vs. bound bacteria) (1). The $K_D$ and $R_T$ were calculated from the Scatchard plot using the following equations: *x*-

intercept = $R_T$ and $K_D$ = $R_T/y$-intercept. All calculations were done using standard statistical methods and graphing was done with the Cricket Graph v1.3.2 program designed for the Macintosh Computer. This program also generated equations for straight lines using the method of least squares.

## C. Results

### I. Development of the Whole Cell Bacterial Adherence Assay.

A number of parameters required standardization in order to develop an assay that would be meaningful and reproducible. These parameters included choice of epithelial cell line and density of the monolayer, bacterial growth conditions, interval of binding, washing conditions for removal of unbound bacteria, and recovery of bound bacteria.

A type II alveolar cell line (19), the A549 human lung pneumocyte cell line, was selected for use in bacterial adherence assays. It was chosen due to its ability to adhere well to plastic during the rigors of an adherence assay and due to the fact that it was a human lung cell line. Since we wished to evaluate the adherence abilities of different strains of *P. aeruginosa*, the organism most often responsible for lung infections in individuals with cystic fibrosis, a human lung cell line was the epithelial cell line of choice. Another factor in favour of the A549 cell line was that it had been used successfully by Chi *et al* in another assay (7). These authors demonstrated that *P. aeruginosa* PAK bound to A549 cells in a dose-dependent manner and calculated an association constant to characterize the interaction. Another human lung cell line (HEL 299) had been tried previously but it was found to detach from a polystyrene surface very easily during normal incubation and wash steps in preliminary assays.

The density of A549 cells used originally in the adherence assay was a confluent monolayer. This became a problem, however, when it was noted that inconsistencies from assay to assay could be due to the fact that all wells in a 24-well culture dish did not necessarily reach equal confluency at the same time. Monolayers that were considered to be confluent gave from 5 x $10^5$ A549 cells per well to 3 x $10^6$ A549 cells per well when trypsinized and counted in a hemacytometer. Overgrown monolayers also contributed to inconsistencies and lower than expected binding results in the adherence assays, probably by binding bacterial cells when only loosely adherent and subsequently detaching during wash steps. It was found empirically that seeding approximately 1 x $10^5$ A549 cells per well and incubating overnight resulted in a monolayer that covered approximately 90% of the well surface, by visual estimation, and was able to be easily reproduced. Adherence assays were performed only with monolayers containing between 8 x $10^4$ A549 cells per well and 2 x $10^5$ A549 cells per well when trypsinized and counted as above.

One of the reasons for using a monolayer rather than buccal epithelial cells (BECs), as described by Irvin *et al*, was that the BECs were 95% non-viable when used in their adherence assays (14). The composition of the surface of these cells would be variable since they would be in various states of viability. A living monolayer could present a more uniform cell surface to the bacteria in a binding assay, resulting in less variability due to surface composition of the epithelial cells. To this end, the viability of an A549 pneumocyte monolayer was assessed by staining with trypan blue. A549 cells used to seed monolayers in 24-well tissue culture plates were diluted 1:1 with 0.4% (w/v) trypan blue and counted in a hemacytometer; viable cells excluded the dye and appeared faint blue to colorless while non-viable cells retained the dye and were stained dark blue. Cells used to seed the monolayers were 98-100% viable. Monolayers were seeded in 6 wells as for an adherence assay, at 1 x $10^5$ A549 cells per well, and allowed to incubate overnight as usual. The following morning, the monolayers were trypsinized, stained and counted as above. Results of the trypan blue counts indicated that the monolayers were, on average, 95% viable. This indicated that the A549 monolayers would likely provide a very uniform cell surface to bacteria in an adherence assay so that different bacterial strains could be compared on the basis of their ability to bind to these monolayers.

The first adherence assays used a bacterial culture that had been grown at 37$^o$C, 250 rpm, to mid-log phase (75 Klett units or 2 x $10^8$ CFU/ml) but this was found to result in very low numbers of adherent bacteria, probably because of pili being mechanically sheared from the bacteria due to the forces generated in the shaking culture. Bacterial growth conditions for the adherence assay were adjusted to the following: an isolated colony was used to inoculate 2 ml of TSB + 5 mM $MgCl_2$ (with antibiotics as required). This culture was grown at 37$^o$C on a tube roller (setting #7) for 6-8 hours, then 1.0 ml was used to inoculate 100 ml of the same media. The 100 ml culture was grown for 16 hours at 37$^o$C, without shaking in order to minimize the number of pili sheared from the bacterial cells during growth. This resulted in a culture that was approximately in mid-log phase at the beginning of the adherence assay. This method of growing the bacteria also resulted in a definite increase in the maximum number of adherent *P. aeruginosa* PAK/A549 cell from 23 CFU/A549 to 56 CFU/A549 (Fig. 3.1).

In order to assess piliation or lack of piliation of *P. aeruginosa* for an adherence assay, some of the inoculum was used to confirm the phage sensitivity profile of the culture being used. A phage sensitivity assay was carried out as described in section 3.B.IV and the result was compared to the known or expected profile for that strain. If it differed from the known profile, that adherence assay was considered invalid.

Two other bacterial controls included in adherence assays were a trypsin control (TC) to ensure that the viability of bacteria was not being affected by the trypsin treatment used to recover bound bacteria, and a growth control (EI, for ending inoculum) to monitor any bacterial growth that might occur over the course of the assay. The TC was carried out as described in section 3.B.III and the number of bacteria recovered from the TC was compared with the number of bacteria originally put into the control. The mean concentrations, as determined by a plate count method, were subject to statistical analysis (T-test) to determine if they were significantly different. On the rare occasions when these means were found to be statistically different, that adherence assay was considered invalid. The most concentrated inoculum and EI mean bacterial concentrations were compared in the same manner, with the same result that when the means were occasionally significantly different, indicating bacterial multiplication over the course of the assay, that assay was considered invalid.

The length of time bacteria were allowed to adhere to A549 monolayers was assessed at 1 hour and 2 hours. After a 2 hour incubation of *P. aeruginosa* PAK at a concentration of 1 x $10^8$ CFU/ml on A549 monolayers, approximately 50% of the cells of the monolayers had rounded up and detached from the bottom of the wells. This indicated that 2 hours was too long for the assay, possibly because *P. aeruginosa* was entering and killing the A549 cells or because *P. aeruginosa*'s extracellular proteases caused the A549 cells to detach from the monolayers. Incubation for 1 hour with up to 2 x $10^9$ CFU/ml resulted in adherent bacteria and monolayers that were still intact and attached to the bottom surface of the wells, so this was chosen as the length of time of incubation to be used for the adherence assay.

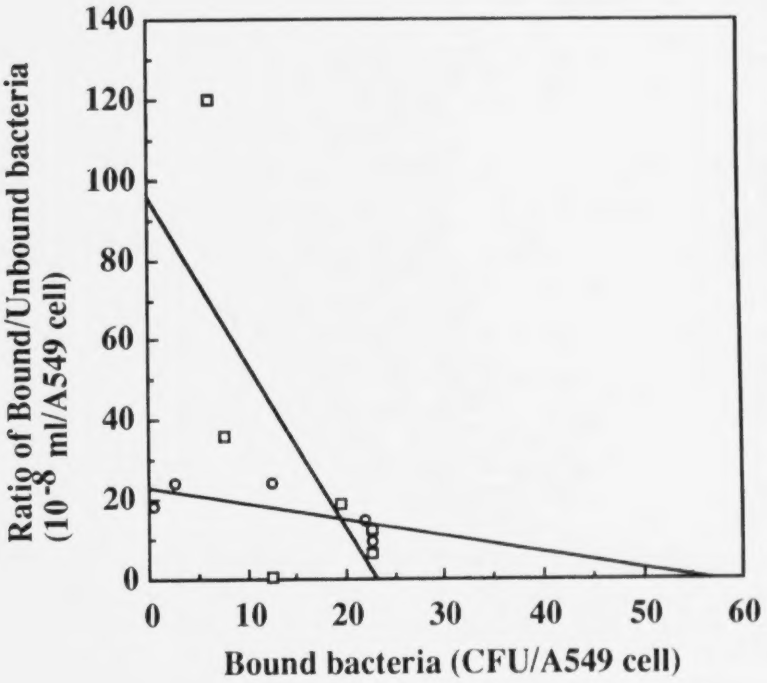


Fig. 3.1. Scatchard plot analysis of binding data from two adherence assays using different bacterial growth conditions. In one case, *P. aeruginosa* PAK was grown at 37°C, 250 rpm to mid-log phase (☐, $r^2$=0.466) before use in the A549 pneumocyte adherence assay. In the second case, an increase in the number of adherent bacteria was seen when PAK was grown for 16 hours at 37°C without shaking (O, $r^2$=0.458).

Wash conditions were another important parameter to optimize since too gentle washing of the monolayers would not remove bacteria that were only weakly bound to the A549 cells, to give artificially high counts, and too vigorous washing would remove bound bacteria to give artificially low counts. Washes were designed using the method of Saiman *et al* (23) as a guide. To determine the number of washes required to remove unbound bacteri to a level below the bound bacteria, 1.7 x $10^9$ CFU/well of *P. aeruginosa* PAK in FC-PBS was added to twelve wells with confluent A549 monolayers, and allowed to incubate at 37°C, 70 rpm for 1 hour. After removal of the inoculum, each well was washed with 0.5 ml FC-PBS for 90 seconds at 37°C, 70 rpm. After the first wash, one set of three wells was trypsinized and the numbers of bound bacteria determined as well as the concentration of bacteria present in the wash. This washing procedure was repeated three more times and numbers of bound bacteria and bacterial concentration in washes from three wells was determined after each wash for a total of four washes. Fig. 3.2 shows a plot of bacterial concentration in washes and bacteria bound to A549 monolayers for each of the four washes. The level of bacteria bound is essentially unchanged, indicating that the washes are not removing bacteria that are firmly bound to the monolayers. The concentration of bacteria dropped 16-fold between the first and second wash and a further 25-fold between the second and third washes. Note that the concentration of the fourth wash was not able to be determined. This indicated that unbound bacteria were being removed to a level that would allow comparison of the numbers of bacteria bound to the A549 monolayers. HBSS with supplements was substituted for FC-PBS as the incubation and wash medium to ensure the viability of the A549 monolayers during the adherence assay when $CO_2$ buffering was not provided. HBSS was designed for equilibration with air (6). The washes were subsequently shortened to 30 seconds each with no change observed in the levels of bound bacteria or wash concentrations of bacteria.

### II. Determination of the Kinetics of Bacterial Binding to A549 Human Lung Pneumocytes.

To determine the kinetics of *P. aeruginosa* PAK binding to A549 pneumocytes, the bacteria were added to monolayers (in triplicate) at a ratio of 110:1 bacteria:A549 cell. This was a lower concentration of bacteria than was normally used for an adherence assay, allowing the survival of the A549 monolayers for over 3 hours. At various time points between 0.5 minute and 3 hours, the inoculum was removed, the wells were washed 4X with HBSS with supplements as described in section 3.C.I, and bound and wash concentrations of bacteria determined by a plate count method on TSB agar. Fig. 3.3 shows the binding kinetics for PAK to A549 human lung pneumocytes. At 110 bacteria to 1 A549 cell, the

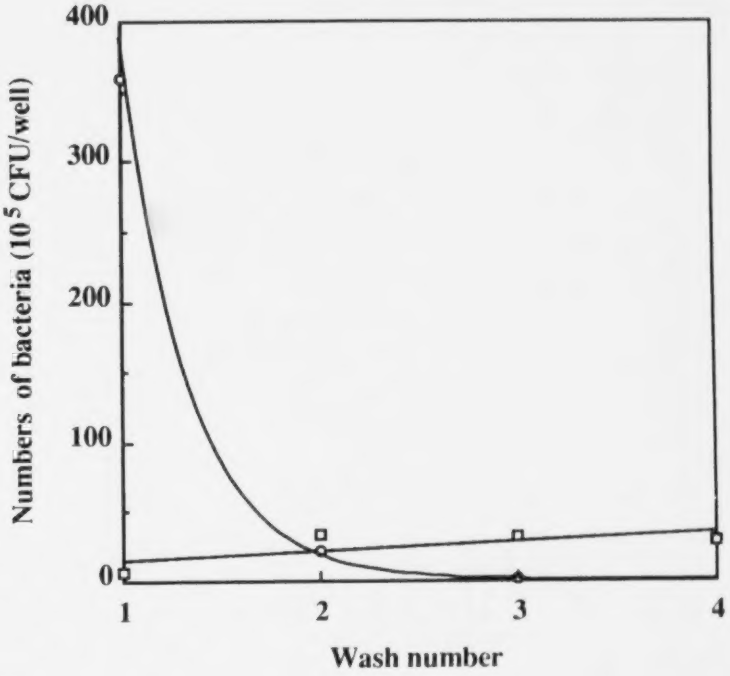


Fig. 3.2. Determination of adherence assay washing conditions for removal of unbound *P. aeruginosa*. *P. aeruginosa* PAK was added to A549 monolayers, allowed to bind for 1 hour, and washed 4 times with buffer as described in section 3.C.I. Numbers of bacteria in each wash (O) and numbers of bacteria bound to the monolayers (☐, $r^2$=0.454) were determined for each wash.

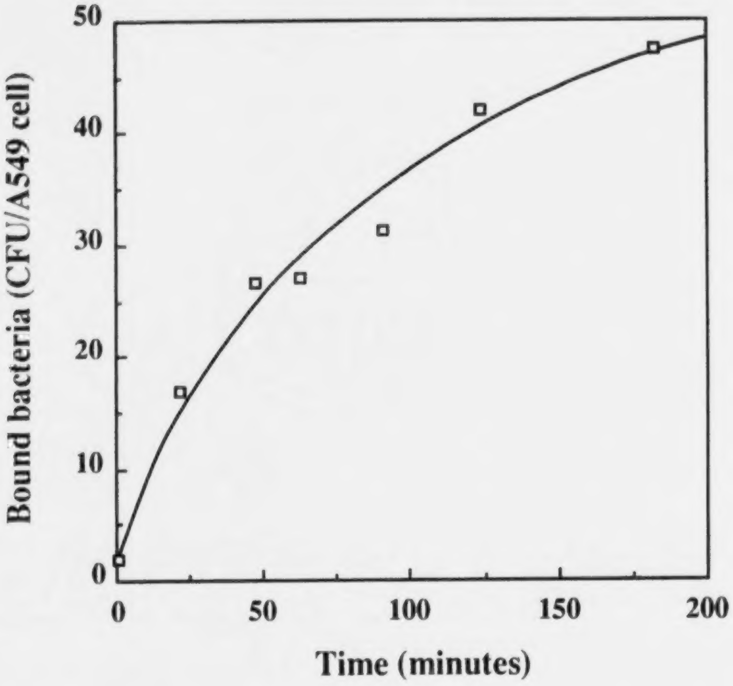


Fig. 3.3. Kinetics of *P. aeruginosa* PAK adherence to A549 human lung pneumocytes. *P. aeruginosa* PAK was incubated on A549 monolayers at a ratio of 110 bacterial cells to 1 A549 cell and the numbers of bacteria bound to the monolayer determined at various time intervals.

level of PAK adherence continually increased over the incubation period and began to level out after about 3 hours. The rate of association of PAK with the A549 monolayer was such that adherent bacteria were detected after only a 0.5 minute incubation, and after 1 hour over half maximum binding was achieved. It was also noted that the rate of adherence was fastest in the first hour of interaction between the bacteria and the monolayer. This reinforced the choice of a one hour incubation as being sufficient to be indicative of the ability of a strain of bacteria to adhere to the A549 monolayers.

### III. Comparison of the Abilities of *P. aeruginosa* PAK and Mutant Strains to Adhere to A549 Human Lung Pneumocytes in a Standard Assay.

In order to determine whether or not the adherence assay would be useful for comparing the adherence abilities of different strains of bacteria, *P. aeruginosa* PAK, *P. aeruginosa* PAK-NP (a mutant in the *pilA* gene), and *P. aeruginosa* PAK-N1 (a mutant in the *rpoN* gene), were subjected to the A549 human lung pneumocyte binding assay. It was expected that the two mutants, neither of which expressed pili, would give much lower numbers of bound bacteria in the adherence assay. As can be seen in Fig. 3.4, binding for PAK was at a much higher level than either PAK-NP or PAK-N1. PAK-NP is a non-piliated mutant in the PAK *pilA* gene (23) and PAK-N1 is a mutant in the PAK *rpoN* gene, which codes for the alternative sigma factor ($\sigma^{54}$) required for the expression of a number of factors in *P. aeruginosa*, including pili, flagella, nitrogen assimilation and degradation genes (16, 27). Fig. 3.5 also shows that while PAK had a dissociation constant ($K_D$) of 4.9 x $10^7$ CFU/ml and a maximum number of receptors per A549 cell of 109, PAK-NP and PAK-N1 appear to be relatively non-specific in their binding as no useful curve could be obtained from their Scatchard analyses.

### IV. Characterization of a *P. aeruginosa* PAO1 Pilin Mutant Retaining the Ability to Assemble Pili.

An interesting chromosomal pilin mutant was constructed by Dr. Mark Farinha by cloning a chloramphenicol acetyltransferase (*cat*) gene into a unique Bam HI site in the disulfide loop coding region of the *P. aeruginosa* PAO1 *pilA* gene and using that construct to replace the wild-type *pilA* gene in PAO1 (Fig. 3.6) (10). This resulted in a fusion that replaced the C-terminal nine codons from *pilA* with eleven new codons from sequence flanking the *cat* gene (Fig. 3.6).

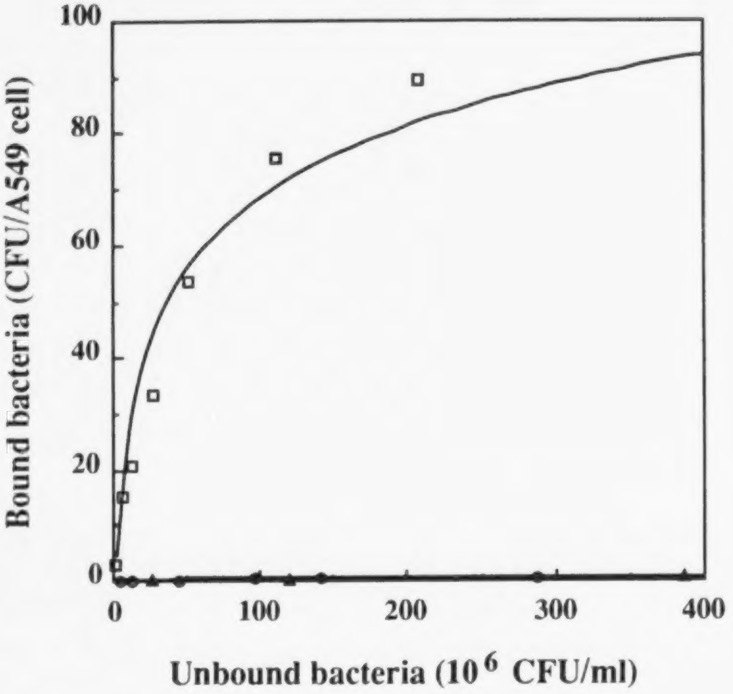
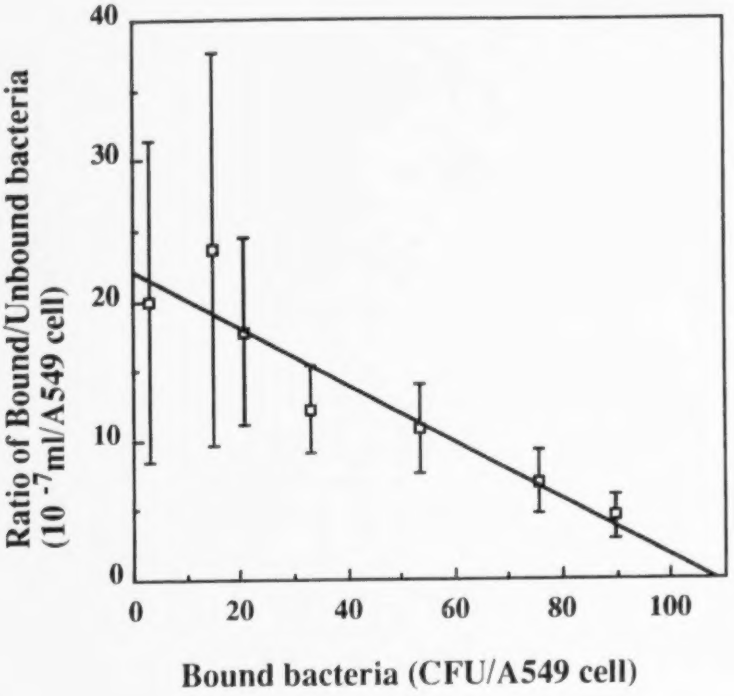


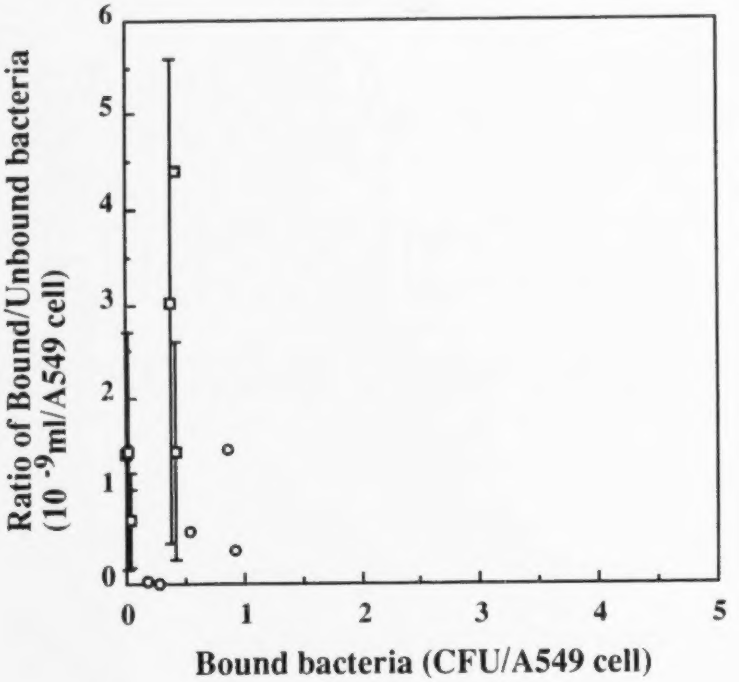
Fig. 3.4. Comparison of the adherence of *P. aeruginosa* PAK (□), PAK-NP (*pilA*) (O) and PAK-N1 (*rpoN*) (Δ)to A549 human lung pneumocytes. A direct plot illustrated the differences in the level of binding due to the presence (PAK) and absence of pili (PAK-NP) and other virulence factors (PAK-N1).

Fig. 3.5. Scatchard plot analysis of *P. aeruginosa* PAK, PAK-NP (*pilA*) and PAK-N1 (*rpoN*) adherence data shown in Fig. 3.4. **A.** *P. aeruginosa* PAK was allowed to adhere to A549 monolayers as described in section 3.B.III and binding was assessed (plot shows the average of two adherence assays, $r^2$=0.885). The total number of bacterial receptors per A549 cell was computed to be 109 and the $K_D$ was 4.9 x $10^7$ CFU/ml. **B.** *P. aeruginosa* PAK-NP and PAK-N1 were allowed to adhere to A549 monolayers as described in section 3.B.III and binding was assessed (plot shows the average of two adherence assays for PAK-NP and a single assay for PAK-N1). Note the difference in scale for the y-axis as compared to **A**. The total number of bacterial receptors per A549 cell and the dissociation constants were unable to be computed from these plots due to the apparently non-specific, low level of adherence of the bacteria to the monolayers.

**A.**


Ratio of Bound/Unbound bacteria
(10 -7ml/A549 cell)
0
10
20
30
40
0
20
40
60
80
100
Bound bacteria (CFU/A549 cell)

**B.**


Ratio of Bound/Unbound bacteria
(10 -9ml/A549 cell)
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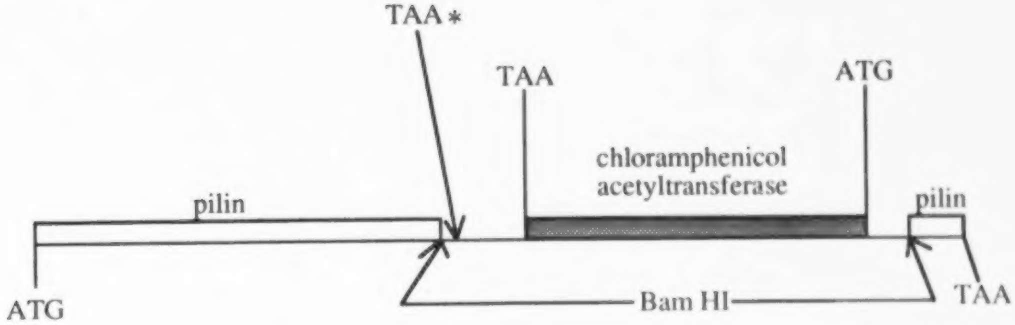
1
2
3
4
5
6
0
1
2
3
4
5
Bound bacteria (CFU/A549 cell)



```
NORMAL PILIN C-TERMINUS

CYS.LYS.SER.THR.GLN.ASP.PRO.MET.PHE.THR.PRO.LYS.GLY.CYS.ASP.ASN.STOP
                        CCG.ATG.TTC.ACT.CCG.AAA.GGT.TGT.GAT.AAC
TGT.AAA.TCT.ACC.CAG.GAT.CC
                     (BamHI)
                          C.GTA.ACC.GTT.TTT.ATC.AGG.CTC.TGG.GAG.GCA.GAA.STOP*
                        PRO.VAL.THR.VAL.PHE.ILE.ARG.LEU.TRP.GLU.ALA.GLU.

MUTANT PILIN C-TERMINUS
```

Fig. 3.6. Strategy used to construct a *P. aeruginosa* PAO1 strain with a modified C-terminus in the *pilA* gene product. The *cat* gene from plasmid pNK2884, which was excised as a Bam HI fragment, was ligated into the disulfide-loop-coding region of the PAO1 *pilA* gene cloned into plasmid pMTL25. The *pilA*::*cat* construct was inserted into the chromosome of *P. aeruginosa* PAO1 via electroporation and subsequent general recombination. *, First in-frame stop codon created by insertion of chloramphenicol acetyltransferase cassette. Figure reproduced with permission from Farinha *et al* (1994), copyright © 1994, American Society for Microbiology (10).

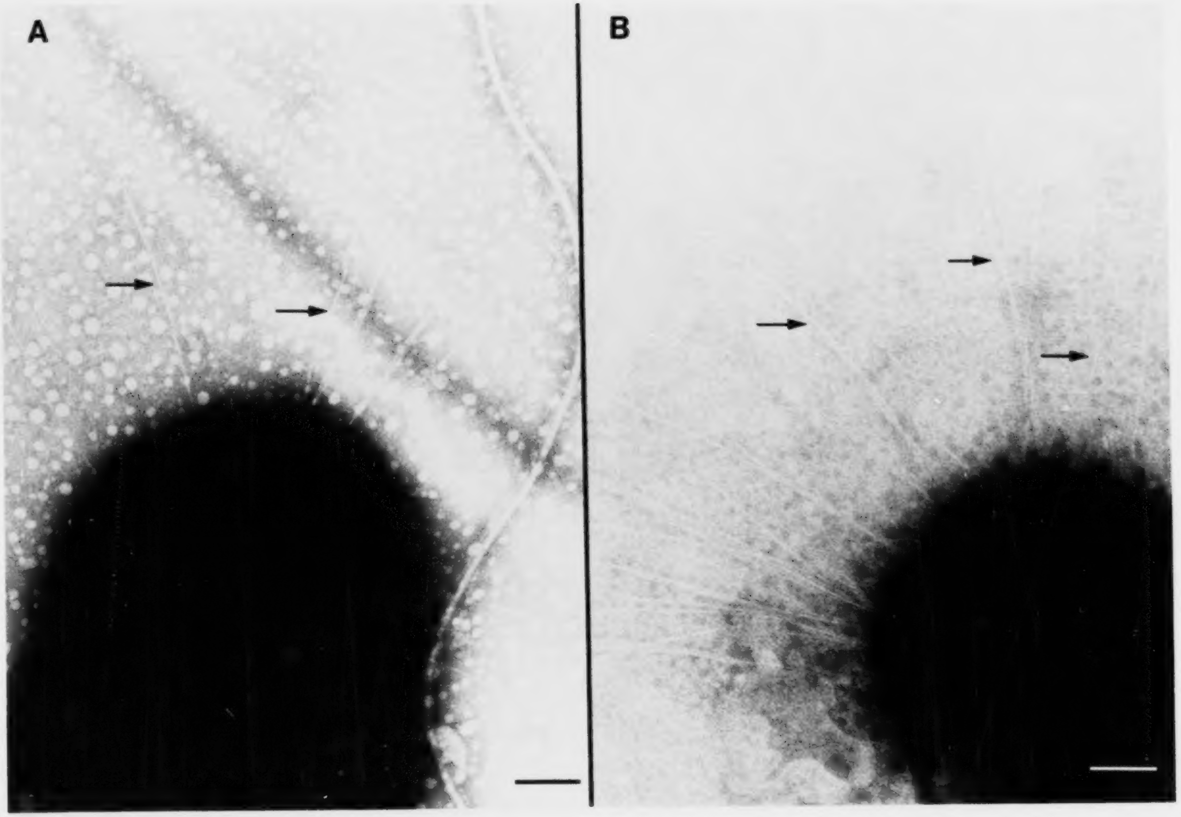
The new amino acid sequence maintained the first seven codons of the *pilA* disulfide loop domain including the first cysteine codon of the loop (CKSTQDP), but the last nine codons of the PilA protein, including the second cysteine of codon of the loop (MFTPKGCDN), were replaced by an amino acid sequence (VTVFIRLWEAE) from pNK2884 (17). The mutant PAO-MP strain was shown to produce pili by both pilus-specific phage sensitivity assay and by electron microscopy. The transmission electron micrographs in Fig. 3.7 show that the polar pili of PAO1 and PAO-MP appeared to be identical in location, number and length. The sensitivity of PAO-MP to pilus specific phages was also determined to be identical to the wild-type PAO1 strain since both strains were sensitive to F116, PO4, and B9 phages but resistant to Pf1 phage. This implied that pili were being assembled normally at the bacterial cell surface and that the retraction function required for phage infection (2, 4) was maintained in the mutant PAO-MP pili.

The pili produced by PAO-MP were then characterized by western immunoblot analysis. PAO1 and PAO-MP whole cell lysates were resolved on SDS-PAGE gels and immune serum specific for PAO pili detected a single band in both PAO1 and PAO-MP with apparent molecular weights of 15.0 kD and 14.5 kD respectively (Fig. 3.8). The weight for PAO1 pilin was in agreement with that previously reported (24), while that for PAO-MP pilin appeared to be smaller despite this protein being two amino acids larger than native PAO1 pilin. This is likely due to the addition of two negatively charged glutamate residues to the PAO-MP PilA protein, which results in slightly faster migration in SDS-polyacrylamide gels (8).

### V. Adherence of *P. aeruginosa* PAO1 and PAO-MP to A549 Human Lung Pneumocyte Monolayers.

Because the PAO-MP mutant appeared to be piliated like its wild-type parent PAO1, a comparison of *P. aeruginosa* PAO1 and PAO-MP binding to A549 cells was carried out in order to assess any differences in the adherence characteristics of the pili. This was done by incubating the bacteria with A549 cells, washing off non-adherent bacteria, and enumerating the bacteria that remained bound to the A549 monolayer. Fig. 3.9 demonstrates that both PAO1 bound to A549 cells in a concentration-dependent manner. Fig. 3.10 shows the Scatchard plot derived using the PAO1 binding data with an apparent dissociation constant, $K_D$, for PAO1 calculated to be 4.0 x $10^7$ CFU/ml. Previously reported results determined the $K_A$ for PAO1 binding to human BECs to be 2.9 x $10^{-8}$ ml/CFU (14). Since $K_D = K_A^{-1}$, the $K_A$ for PAO1 determined by these experiments was 2.5 x $10^{-8}$ ml/CFU, which is in agreement with the PAO1-BEC $K_A$. The data indicated

Fig. 3.7. Negatively stained preparations of *P. aeruginosa* PAO1 (A) and PAO-MP (B) from Farinha *et al* (1994) (10). The arrows indicate polar pili. The bar represents 100 nm. Figure reproduced with permission from Farinha *et al* (1994), copyright © 1994, American Society for Microbiology (10).


A
B

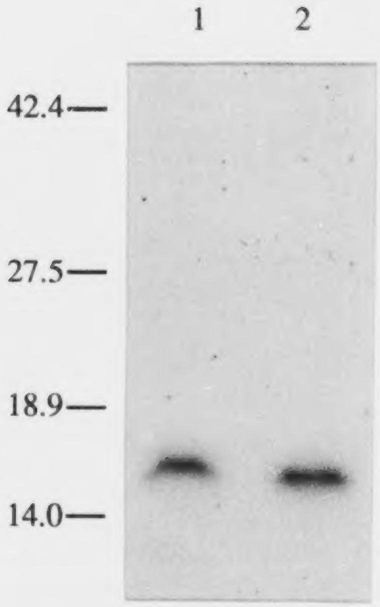


Fig 3.8. Western immunoblot analysis of pilin protein from *P. aeruginosa* PAO1 (lane 1) and PAO-MP (lane 2). Equal volumes (20 μl) of whole-cell-lysate preparations were electrophoresed on a 12.5% SDS-polyacrylamide gel and the pilin proteins were detected with whole rabbit serum against PAO1 that had been preadsorbed with a PAO1 strain unable to produce pilin. Bound antibody was detected with $^{125}$I-labeled protein A from *Staphylococcus aureus*. Molecular weight standards (kDa) are shown to the left.

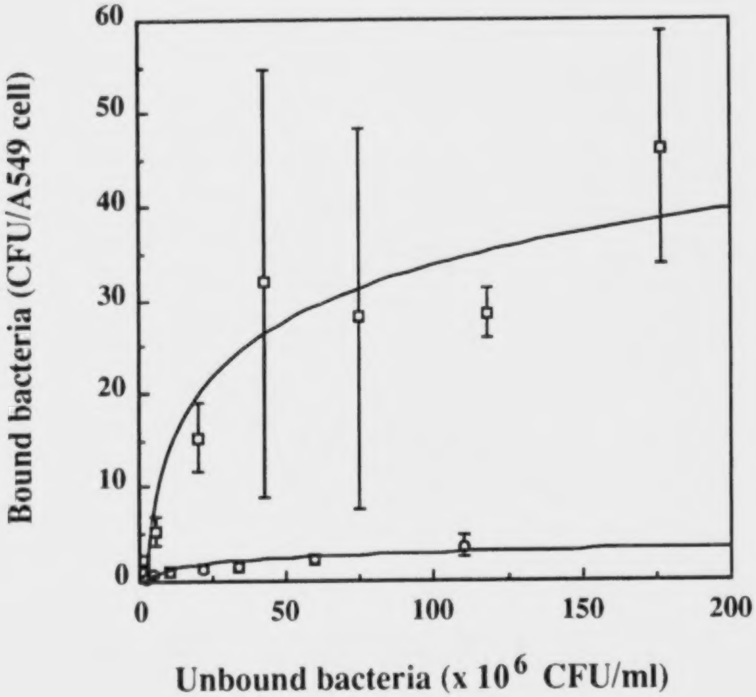


Fig. 3.9. Saturation curve (direct plot) of *P. aeruginosa* PAO1 (□) and PAO-MP (O) adherence to A549 cells from Farinha *et al* (1994) (10). Increasing concentrations of each strain were incubated on an A549 pneumocyte monolayer with approximately $10^5$ A549 cells per well of a 24-well plate. The monolayers were washed, trypsinized, and assayed for adherent bacteria as described in section 3.B.III. Figure reproduced with permission from Farinha *et al* (1994), copyright © 1994, American Society for Microbiology (10).

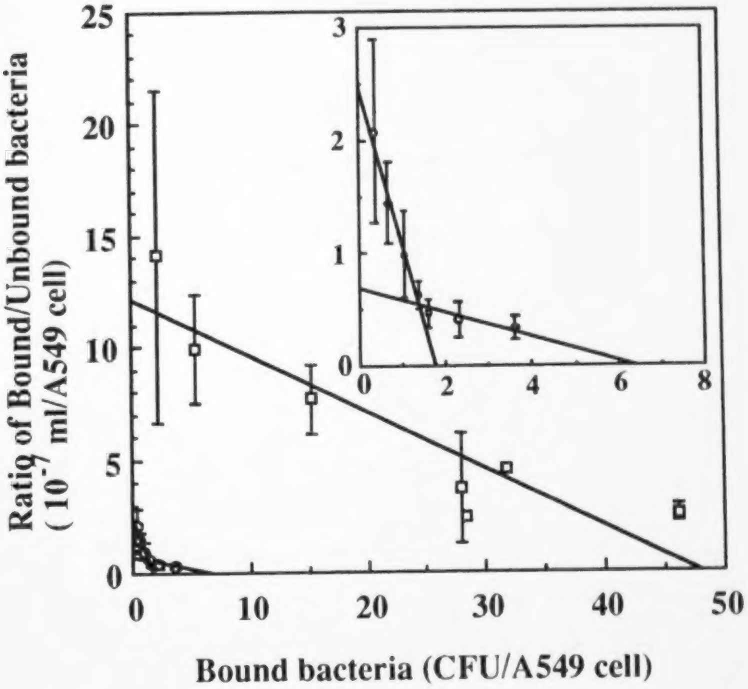


Fig 3.10. Scatchard plot analysis of *P. aeruginosa* PAO1 and PAO-MP adherence data shown in Fig. 3.9. *P. aeruginosa* PAO1 (□, $r^2$=0.822) and PAO-MP (O, high affinity system $r^2$=0.974; low affinity system $r^2$=0.743) adherence to A549 human lung pneumocytes was analyzed by the method of least-squares regression to calculate the total receptors per A549 pneumocyte and the dissociation constant for each strain. The inset is an enlarged view of the PAO-MP plot.

that approximately 50 receptors per A549 cell were available for binding of piliated PAO1 cells under the conditions used in this study. Fig. 3.9 also shows PAO-MP binding to A549 cell monolayers. The level of adherence of PAO-MP was approximately 10-fold lower than that measured for its parent, PAO1. The Scatchard plot of PAO-MP (Fig. 3.10) indicated the possible existence of at least two other adherence systems that became evident only in the absence of pili binding. It was not determined whether one of these represented the altered PAO-MP pili or whether both characterized non-pilus adherence systems. The $K_D$ values for these high and low affinity systems were determined to be 7.3 x $10^6$ CFU/ml and 9.2 x $10^7$ CFU/ml respectively. The total receptors for each were 6 per A549 cell for the low affinity system and 2 per A549 cell for the high affinity system.

### VI. Virulence of Wild-Type and Mutant Strains of *P. aeruginosa* PAK and PAO1 in the A.BY/SnJ Mouse Infection Model.

Separate challenges were performed on A.BY/SnJ mice with a number of *P. aeruginosa* strains including mutant and wild-type PAO1 and PAK strains. This strain was used because it was not necessary to immunosuppress the mice with burn treatment or cyclophosphamide to demonstrate virulence for *P. aeruginosa*. The natural $LD_{50}$ values were low enough in this mouse strain to demonstrate statistically significant increases in $LD_{50}$ values when less virulent strains of *P. aeruginosa* were tested. Table 3.2 shows that the wild-type PAO1 strain ($LD_{50}$ = 7.0 x $10^4$ bacteria per mouse) was approximately 5-fold more virulent than the wild-type PAK strain ($LD_{50}$ = 3.8 x $10^5$ bacteria per mouse). When the pilus adhesin was absent in the non-piliated PAK strains (PAK-NP; PAK-*rpo*N), or modified in the PAO1 strain (PAO-MP), the $LD_{50}$ value increased in all cases to 2-3 x $10^6$ bacteria per mouse (Table 3.2), as would be expected from strains unable to express an important virulence factor such as pili. The $LD_{50}$ values for PAO1 vs. PAO-MP ($T_{calc}$=14.5), PAK vs. PAK-NP ($T_{calc}$=14.5), and PAK vs. PAK-N1 ($T_{calc}$=5.26), were all found to be significantly different at the 95% confidence limit by the T-test for significant difference of means.

Table 3.2. $LD_{50}$ comparison for *P. aeruginosa* strains using the A.BY/SnJ mouse model.

| *P. aeruginosa* strain | $LD_{50}$[a] | Standard error[a] | # assays[b] |
|---|---|---|---|
| PAK | $3.8 \times 10^5$ | $\pm 1.7 \times 10^5$ | 15 |
| PAK-NP | $1.4 \times 10^6$ | $\pm 1.6 \times 10^5$ | 9 |
| PAK-N1 | $9.0 \times 10^5$ | $\pm 2.0 \times 10^5$ | 4 |
| PAO1 | $7.0 \times 10^4$ | $\pm 2.5 \times 10^4$ | 17 |
| PAO-MP | $2.5 \times 10^6$ | $\pm 7.0 \times 10^5$ | 10 |

[a] $LD_{50}$ and standard error were calculated using the probit method (29). Units are CFU/mouse.

[b] Replicate assays included 8 or 10 mice/assay.

## D. Conclusions

The development of a whole cell adherence assay facilitated the comparison of the abilities of different strains of bacteria to bind to a monolayer of A549 human lung pneumocytes. This allowed characterization of each adherence system through graphical analysis based on the rules outlined for Clark's Occupancy Theory (1), as discussed in section 3.A.II. The adherence abilities of *P. aeruginosa* PAK and two non-piliated mutants were compared using the A549 binding assay. It was obvious from the Scatchard plots (Fig. 3.4) that the level of binding of the *pilA* and *rpoN* mutants was low and apparently non-specific compared to that for PAK. It was interesting that the levels of binding of PAK-N1 (*rpoN* mutant) and PAK-NP (*pilA* mutant) were similar since PAK-N1 lacked expression not only of pili, but also of flagella and other diverse gene products. This suggests that pili play a major role in *P. aeruginosa* adherence to A549 cells.

Some disadvantages of this adherence assay were that it did not distinguish between adherent and internalized bacteria and that it was subject to error in measurements because of the nature of the system. This error was undoubtedly due, in large part, to the difficulty of enumerating bacteria bound to epithelial cells using a plate count method. The large error bars in the Scatchard plots indicate that it is difficult to remove all the bound bacteria from the surface of the A549 cells, therefore, it is unlikely that all single bacterial colonies in the "Bound Bacteria" plate count will have arisen from a single cell.

Another concern is that even though pili are the virulence factor responsible for most of the bacterial adherence, they are not the only adhesin present on *P. aeruginosa*. A whole cell adherence assay potentially involves the interaction of many adhesins to produce an effect that is measured as adherence of the whole bacterial cell. It is important to remember this when interpreting the results from binding assays. Advantages of this system were that adherence to viable epithelial cells was measured and only viable bound bacteria were counted since enumeration was accomplished by a plate count method. The similar $K_D$'s obtained for this adherence assay and the previously reported BEC adherence assay indicated that the A549 cell assay was comparable to the BEC assay in the demonstration of *P. aeruginosa* adherence.

Bacterial adherence is important as the first step in establishment of an infection. A comparison of the adherence ability of *P. aeruginosa* PAO1 with the pilin mutant PAO-MP was undertaken in order to investigate the central role that pili play in adherence to epithelial cells. This had been demonstrated previously (9) but their relative importance compared to other adhesins such as flagella or exoenzyme S had not been well defined. The construction of the PAO-MP pilin mutant was carried out in order to remove a portion of the C-terminal disulfide loop of the pilin protein without interfering with the integrity and non-adhesive functions of pili. This allowed a direct assessment of the contribution of the disulfide loop, which had been demonstrated *in vitro* to be the adhesive epitope of the pilin protein (18), to whole cell binding to epithelial cells.

A549 human lung pneumocytes were shown to express approximately 50 receptors for PAO1, 42 of which (approximately 80%) were shown to be for pili. The remainder of available sites for all other non-pilus adhesins appeared to be made up of at least two other binding systems which were evident from the Scatchard plot (Fig. 3.9) once the predominant binding activity of the pilus adhesin was removed in the PAO-MP mutant. Although the altered PAO-MP pilin subunit assembled into pili that retained their normal phage sensitivity and appeared to be indistinguishable from the wild-type pili of PAO1, these mutant pili were not able to bind to A549 cells due to the disruption of the adherence domain in the disulfide loop of the pilin protein. PAO-MP clearly demonstrated that the amino-acid sequence near the C-terminus of pilin was not involved in pilin assembly, structural integrity or non-adherent functions such as phage binding or pilus retraction.

When the virulence of these strains was tested, PAO1 produced a 42X lower $LD_{50}$ value than the altered PAO-MP mutant in the A.BY/SnJ mouse model. PAO-MP was observed to be as virulent as the non-piliated PAK-NP (*pilA*) and the PAK-N1 *rpoN* mutant, which lacked pili, flagella and exoenzyme S adhesins as well as several other virulence factors which depend on RpoN directed transcription (27). Non-pilus adhesins apparently do not contribute significantly to *P. aeruginosa* virulence since the lack of expression of non-pilus adhesins as well as the pilus adhesin in the PAK-*rpo*N strain did not increase the $LD_{50}$ value over that seen when only the pilus adhesin was absent, as in PAK-NP and PAO-MP. The conclusion drawn from these experiments was that the pilus adhesin was the principal adherence-related virulence factor in *P. aeruginosa* infection.

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# Chapter 4

## Studies on *pilG*, a Gene Involved in *Pseudomonas aeruginosa* Pilus Assembly

# A. Introduction

## I. Background Information.

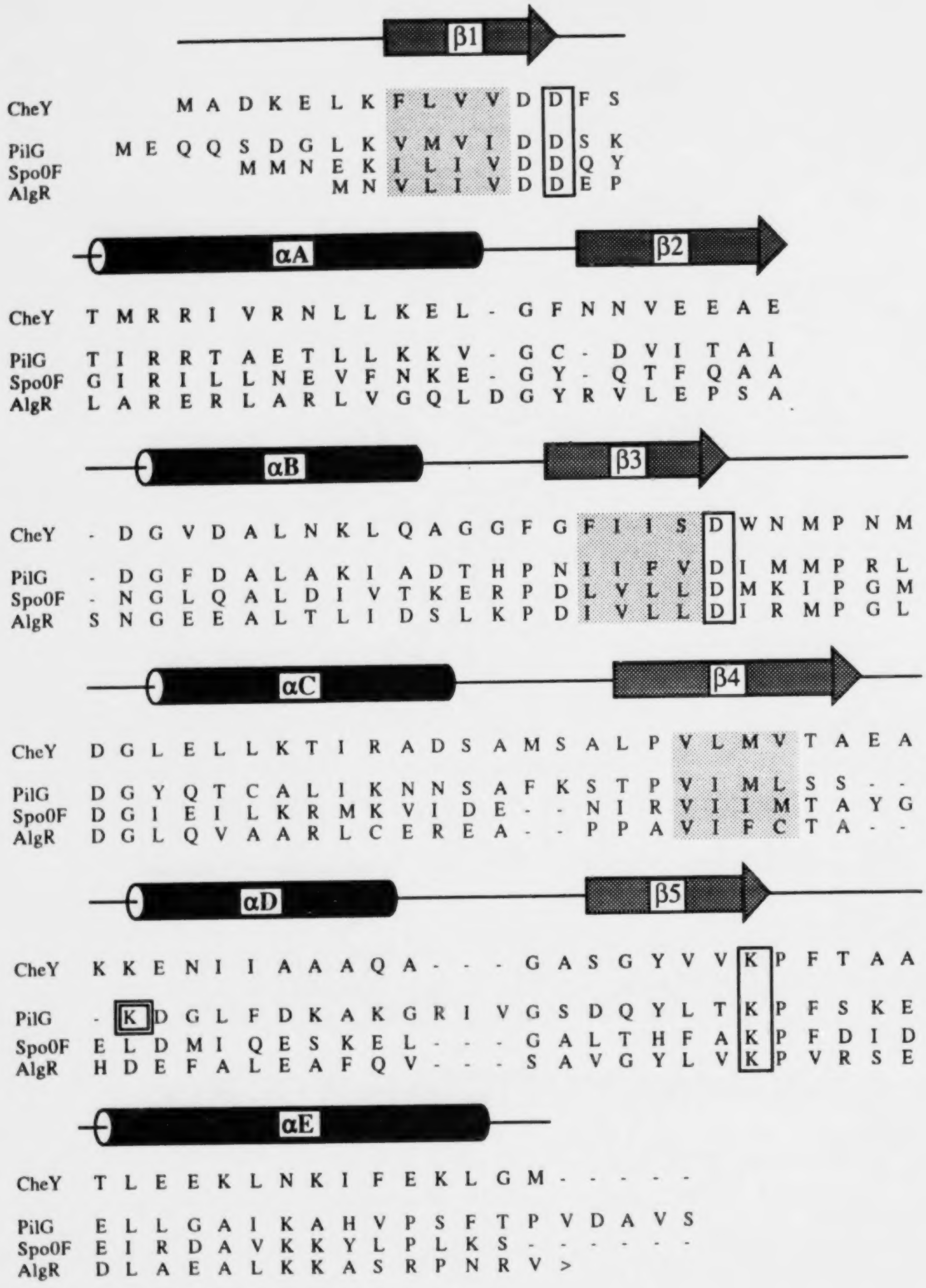
Type IV pili confer a number of characteristics on the bacteria that produce them. Specifically with *P. aeruginosa*, type IV polar pili allow the bacteria to adhere to surfaces such as epithelial cells which plays an important role in virulence. Type IV pili may also provide a mode of entry into the bacterial cell for pilus-specific phages. These have been proposed to attach to pili and accomplish infection when pulled close to the cell surface by pilus retraction (3, 6). Pili also provide a form of surface-associated locomotion termed twitching motility (5, 15, 16, 42). As outlined in chapter 1, pili are constructed of pilin protein subunits arranged in a helical array and anchored in the membrane of the bacterium by an unknown mechanism while "pools" of pilin in the membranes serve as the source of the protein. While the mechanism of pilus assembly is as yet unknown, what is known is that it is a complicated process involving the coordination of protein processing, transport, and signalling, and it requires the expression of a relatively large number of genes (43).

Some of the genes necessary for pilus expression and assembly in *P. aeruginosa* have been identified. Chapter 1 outlined the roles of a number of these genes, including a group of genes involved in signal transduction. One of these, the *pilG* gene, was identified by Darzins in *P. aeruginosa* PAO1 when a spontaneous mutant gave rise to a phenotype that was resistant to one of three pilus-specific bacteriophages (8). This mutant remained sensitive to two of the three phages but was incapable of twitching motility. This indicated that a pilus might be present to allow some sensitivity to pilus-specific phages, but obviously the bacteria were not producing a fully functional pilus. The gene causing this mutant phenotype was identified by complementation of the mutation as *pilG* and subsequently the wild-type *pilG* gene was sequenced. The predicted amino acid sequence of the PilG protein shared significant similarity with a group of bacterial proteins known as response regulators (RRs), particularly a subclass of RRs that consisted only of the single N-terminal RR domain which had kinase activity but no DNA-binding activity (Fig. 4.1). Darzins also reported that PilG was found to have 32% amino acid identity with the well-characterized enteric chemotaxis protein CheY (8, 22, 28, 30, 31).

## II. Signal Transduction and the Importance of CheY.

The CheY protein is part of the signal transduction system that mediates chemotaxis in *Escherichia coli* and *Salmonella typhimurium* (35). When a ligand for the chemosensory pathway, such as aspartate, binds to its specific receptor in the bacterial inner membrane

Fig. 4.1. Structural features and sequence alignment of *Salmonella typhimurium* CheY with three other response regulator proteins: PilG and AlgR from *P. aeruginosa* and Spo0F from *Bacillus subtilis* (8, 34). Several structural features of CheY are conserved among the other response regulators: shaded residues indicate hydrophobic core regions of CheY, and three residues that are highly conserved among all response regulators are boxed. These residues, Asp-13, Asp-57, and Lys-109, are located at or near the phosphorylation site of CheY. The double-boxed residue indicates Lys-90 of PilG which was changed to Glu-90 in the PilG point mutant FA2. Shaded arrows represent the five parallel β-strands that make up the core β-sheet and cylinders represent the five α-helices that surround the β-sheet.


β1
CheY M A D K E L K F L V V D D F S
PilG M E Q Q S D G L K V M V I D D S K
Spo0F M M N E K I L I V D D Q Y
AlgR M N V L I V D D E P
αA
β2
CheY T M R R I V R N L L K E L - G F N N V E E A E
PilG T I R R T A E T L L K K V - G C - D V I T A I
Spo0F G I R I L L N E V F N K E - G Y - Q T F Q A A
AlgR L A R E R L A R L V G Q L D G Y R V L E P S A
αB
β3
CheY - D G V D A L N K L Q A G G F G F I I S D W N M P N M
PilG - D G F D A L A K I A D T H P N I I F V D I M M P R L
Spo0F - N G L Q A L D I V T K E R P D L V L L D M K I P G M
AlgR S N G E E A L T L I D S L K P D I V L L D I R M P G L
αC
β4
CheY D G L E L L K T I R A D S A M S A L P V L M V T A E A
PilG D G Y Q T C A L I K N N S A F K S T P V I M L S S - -
Spo0F D G I E I L K R M K V I D E - - N I R V I I M T A Y G
AlgR D G L Q V A A R L C E R E A - - P P A V I F C T A - -
αD
β5
CheY K K E N I I A A A Q A - - - G A S G Y V V K P F T A A
PilG - K D G L F D K A K G R I V G S D Q Y L T K P F S K E
Spo0F E L D M I Q E S K E L - - - G A L T H F A K P F D I D
AlgR H D E F A L E A F Q V - - - S A V G Y L V K P V R S E
αE
CheY T L E E K L N K I F E K L G M - - - - -
PilG E L L G A I K A H V P S F T P V D A V S
Spo0F E I R D A V K K Y L P L K S - - - - - -
AlgR D L A E A L K K A S R P N R V >

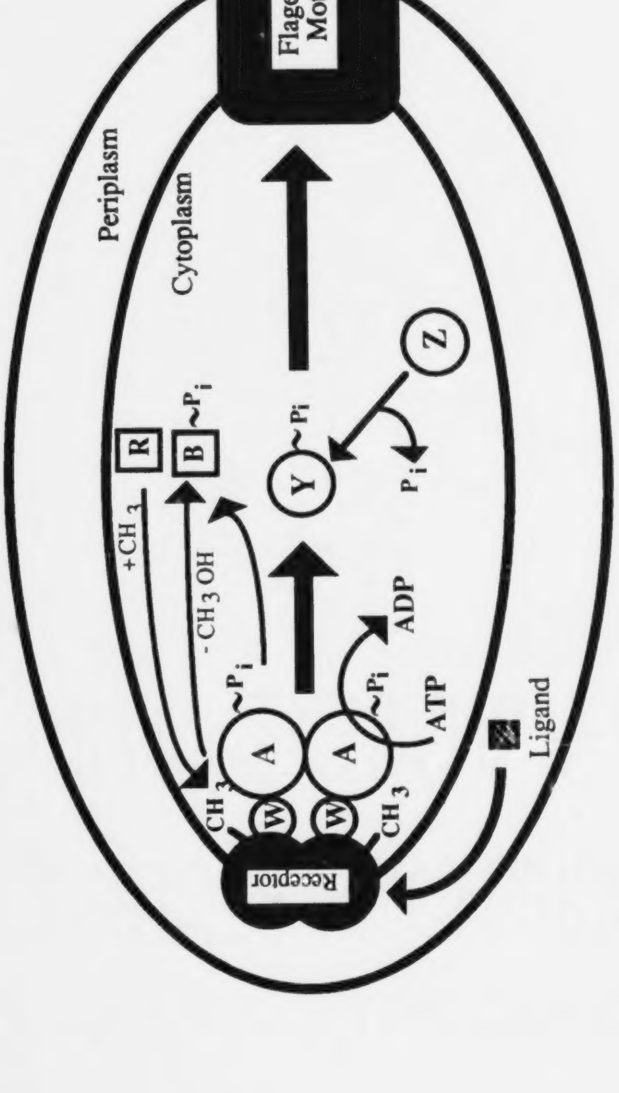
(see Fig. 4.2), this signal is passed through CheW, an auxiliary protein that forms a ternary complex with the receptor and the histidine protein kinase CheA, to CheA whose kinase activity catalyzes the transfer of a $\gamma$-phosphoryl group from ATP to the N3 position of one of its own histidine residues. The CheY protein then catalyzes its own phosphorylation by the transfer of the phoshoryl group from CheA and when phospho~CheY binds to switch components at the flagellar motor, it causes tumble swimming responses by regulating switching of the direction of flagellar rotation. This signalling pathway is modulated by three additional enzymes: the CheR methyltransferase, the CheB methylesterase, and CheZ, a phosphatase-activating protein. CheZ modulates the activity of CheY, probably either by catalyzing the direct hydrolysis of the phospho-aspartyl side-chain or by acting allosterically to stimulate the known auto-dephosphorylation activity of CheY. The CheB methylesterase, like CheY, uses CheA as a source of phosphoryl groups and phospho~CheB, together with CheR, controls the state of methylation of the receptor. It is the fluctuations in the methylation of the receptor that provide a feedback mechanism to control the function of the active receptor-CheW-CheA ternary complex and, therefore, chemotaxis (9, 32, 40).

### III. Comparison of PilG and CheY Structural and Functional Motifs.

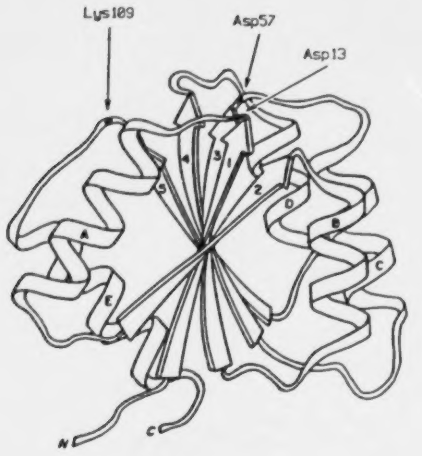
The idea that the PilG protein could play a role similar to CheY, but in the assembly of pilin monomers into pili, is supported by the fact that the two proteins share a high degree of apparent sequence and functional domain similarity. As previously noted, the CheY protein plays a role in the signal transduction cascade governing the chemotactic responses of enteric bacteria by functioning as the response regulator of the chemotaxis system. It is possible that PilG could perform a similar function in the signal transduction pathway leading to the assembly of pili in *P. aeruginosa*. The CheY protein and other homologues, including PilG, have conserved structural motifs corresponding to three of the five strands making up the parallel $\beta$-sheet at the core of the CheY molecule (Fig. 4.1 and Fig. 4.3) (8, 34). These three motifs are short stretches of four hydrophobic amino acids that make up part of strands $\beta1$, $\beta3$, and $\beta4$ of CheY. These structures are important in providing the proper conformation of the active site and the invariant Asp-13 and Asp-57 residues that are important for active site function occur at the carboxyl-termini of the $\beta1$ and $\beta3$ strands, respectively. Conservation of these motifs in PilG suggests structural similarities between PilG and CheY.

There are similarities between CheY, PilG, and other response regulators in function as well as structure. Amino acid residues that are highly conserved among the response

Fig. 4.2. A representation of the chemosensory pathway of *E. coli* and *S. typhimurium* adapted from Falke *et al* (1995) (11). Activation of a specific receptor by its ligand results in the activation of a cytoplasmic histidine kinase pathway composed of proteins encoded by the *che* genes. CheW couples the receptor to the kinase, CheA, to provide a stable receptor-kinase complex. CheA autophosphorylates and the phosphate is transferred to a response regulator, either CheY or CheB. Phospho~CheY regulates switching of the flagellar motor while phospho~CheB controls methylation of the receptors along with CheR. CheZ facilitates the dephosphorylation of CheY (11). Abbreviations: **A**, CheA; **B**, CheB; **R**, CheR; **W**, CheW; **Y**, CheY; **Z**, CheZ; **P$_i$**, phosphate.


Periplasm
Cytoplasm
Flagellar Motor
Receptor
W
W
CH 3
CH 3
A
A
~P i
~P i
ATP
ADP
Ligand
+CH 3
- CH 3 OH
R
B ~P i
Y ~Pi
Z
P i

**A**


Lys109
Asp57
Asp13
4
3
1
5
2
A
D
B
C
E
N
C

**B**

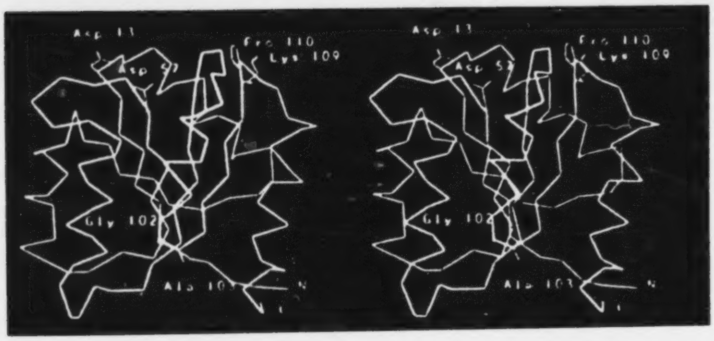

Asp 13
Pro 110
Lys 109
Asp 57
Gly 102
Ala 103
N
C
Asp 13
Pro 110
Lys 109
Asp 57
Gly 102
Ala 103
N
C

Fig. 4.3. Tertiary structure of the enteric CheY protein. **A.** Standard X-ray crystallographic techniques have been used to determine the structure of CheY to 2.7 Å resolution, showing it to be a doubly-wound α/β protein that consists of five α-helices surrounding a five-stranded parallel β-sheet in this structure from J. B. Stock *et al* (32). The highly conserved Asp-13, Asp-57, and Lys-109 residues important for the active site conformation and activity are shown. Reproduced, with permission, from the Annual Review of Biophysics and Biophysical Chemistry, Volume 20, © 1991, by Annual Reviews Inc. **B.** Stereo image of CheY from A. M. Stock *et al* (33) that shows the positions of six residues (Asp-13, Asp-57, Gly-102, Ala-103, Lys-109, Pro-110) that are conserved in the CheY family of bacterial regulatory proteins. Note the proximity of Asp-13, Asp-57, Lys-109, and Pro-110 at the active site of CheY where Asp-57 has been shown to accept a phosphoryl group from CheA (28). Reprinted with permission from Nature, Volume 337:745-749, copyright © 1989, Macmillan Magazines Limited.

regulators include Asp-12, Asp-13, Asp-57, and Lys-109 and these residues all play a role in the active site of CheY (Fig. 4.3). All four residues occur on the same face of the molecule, in the loops between the β-strands and α-helices and all have been shown to help form an acidic pocket that makes up the phosphorylation site. Asp-12, Asp-13, and Asp-57 participate in the chelation of a divalent magnesium ion required for phosphate transfer from CheA to CheY (22) and Lys-109 may play a role in either the acid-catalyzed cleavage of the CheA histidine phosphate necessary for phosphate transfer to CheY or it may participate in the formation of a salt bridge with Asp-57 that is responsible for the conformational change that would occur on phosphorylation of CheY (28). Fig. 4.4 shows the octahedrally coordinated Mg(II) and the active site residues involved (35). Asp-57 accepts the phosphate group transferred from CheA (28), probably by a mechanism involving a restructuring of the active site as the Mg(II) likely shifts to coordinate with the phosphate oxygens of the transferred group (35). The Mg(II) is required for both phosphorylation and dephosphorylation of CheY. The high degree of conservation of the amino acid residues around the active site points to a requirement for a specific structural conformation that allows CheY to carry out its function as a response regulator. Conservation of these residues in PilG indicates that this protein probably also requires a very specific conformation for function. Although phosphorylation of PilG has not been demonstrated, the occurrence of conserved structural and functional motifs between PilG and CheY suggests that the structure and function of the PilG protein in pilus assembly may be similar to that of CheY. In the following experiments, the *pilG* mutants isolated and constructed by Darzins were further characterized from the perspective of discovering more about the role of PilG in pilus assembly and in virulence in *P. aeruginosa*. The kind gift of the spontaneous and insertion *pilG* mutants from Dr. Aldis Darzins provided the opportunity to study the effects of these mutations.

## B. Materials and Methods

### I. Strains, Culture Conditions and Plasmids.

The strains and plasmids used in these studies are listed in Table 4.1. Note that strains or plasmids in parentheses are included for discussion purposes only and were not used in experiments. *P.aeruginosa* strains were maintained on Tryptic Soy Broth (TSB) agar plates (agar added at 1.5% (w/v)). Media for *P. aeruginosa* strains was supplemented as needed with kanamycin (50 μg/ml) or tetracycline (100 μg/ml), and media for *Escherichia coli* strains was supplemented as needed with ampicillin (100 μg/ml), kanamycin (35 μg/ml), or tetracycline (10 μg/ml).

Fig. 4.4. Octahedral coordination of Mg(II) at the active site of CheY. Mg(II) is directly coordinated at the active site of CheY by the carboxylate oxygens of Asp-13 and Asp-57, and the backbone carboxyl of Asn-59. Asp-12 interacts through a water molecule, and two other water molecules participate in the coordination of the metal. The view presented is derived from X-ray crystallographic data at 0.17 nm resolution obtained and interpreted by Stock et al (35).

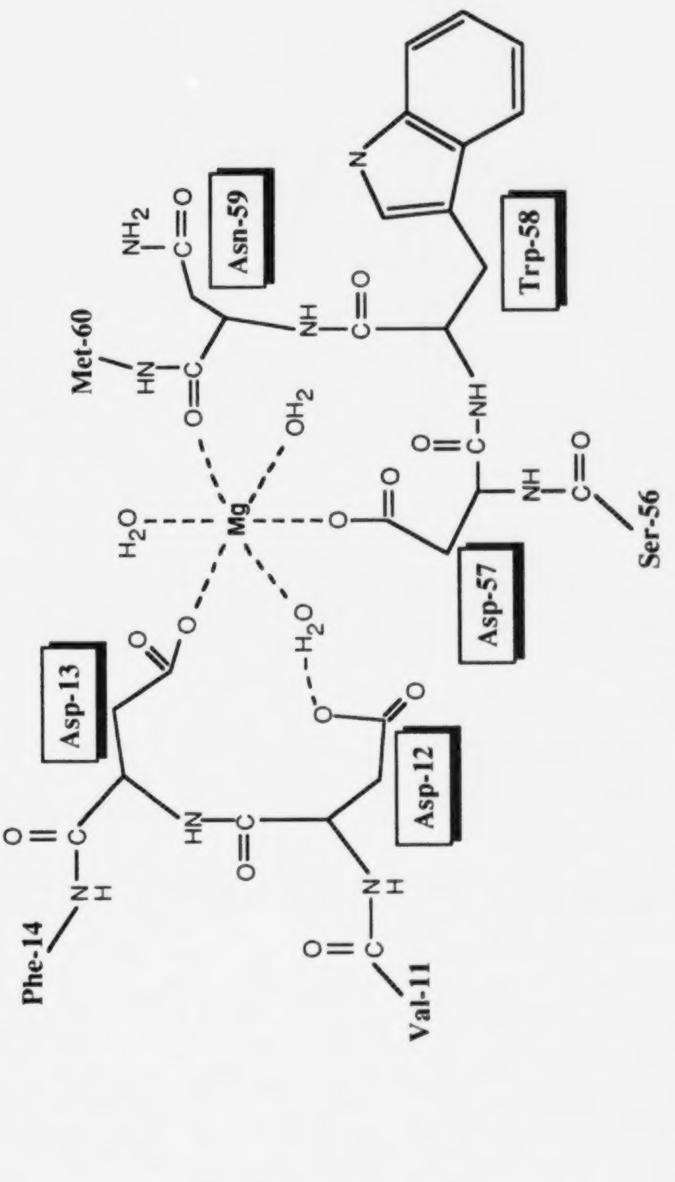

Phe-14
Asp-13
Asp-12
Val-11
Mg
H2O
OH2
Asp-57
Ser-56
Trp-58
Asn-59
Met-60
NH2

Table 4.1. Bacterial strains and plasmids used in this study.

| Strain, phage or plasmid | Genotype or description[a] | Source or reference |
|---|---|---|
| *E. coli* | | |
| DH5α | φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 phoA hsdR17*($r_K^-$, $m_K^+$) *supE44* λ$^-$ *thi-1 gyrA96 relA1* | Gibco BRL Life Technologies |
| *P. aeruginosa* | | |
| BLP3 | Tn501 *pilA* mutant in PAO1 | |
| FA2 | PAO1*pilG1* | (8) |
| FA3 | PAO4141*pilG*::mγ∂-1 Km$^r$ | (8) |
| PAO1 | Prototroph, FP$^-$ | (17) |
| PAO4141 | *pro-9024 met-9020 blaP9202 blaJ9111 aph-9001* FP$^-$ | (8) |
| Phages (pilus-specific) | | |
| B9 | noncontractile tailed phage | R. E. W. Hancock |
| F116 | temperate DNA transducing phage | (25, 29) |
| Pf1 | filamentous phage | (7, 36) |
| PO4 | noncontractile tailed phage | (4, 6) |
| Plasmids | | |
| (pADD698) | 6.2 kb *pilG*-containing fragment in pCP13 vector, Tc$^r$ | (8) |
| pBluescript SK(+) | cloning vector, Ap$^r$ | Stratagene |
| pBPG-01 | 0.7 kb *pilG*-containing fragment from FA2 in pBluescript SK(+), Ap$^r$ | this study |
| pBPG-21 | 0.7 kb *pilG*-containing fragment from PAO4141 in pBluescript SK(+), Ap$^r$ | this study |

[a] Ap$^r$, ampicillin resistance; Tc$^r$, tetracycline resistance; *P. aeruginosa* gene designations are as described by Royle et al. (24).

## II. Motility Assays.

Motility and twitching motility assays were carried out essentially as described by Darzins (8). L-broth (LB) agar plates for both assays were poured to a depth of approximately 5 mm and dried at room temperature for 6-7 hours. Flagellar motility was tested by stab-inoculating soft (0.3% (w/v)) LB agar with a strain, to the bottom of the polystyrene dish. Plates were incubated for 12 hours at 37$^o$C in an open plastic bag to prevent drying of the medium and radial zones of motility visualized between the agar and the petri dish were measured.

Flagellar motility was also confirmed by observing a wet mount by phase contrast microscopy. Each strain was grown from a single colony in 10 ml of TSB at 37°C, overnight, without shaking. A single drop of culture was placed on a glass microscope slide, covered with a coverslip, and observed at 1000X under oil using phase contrast.

Twitching motility was tested on LB agar (1%) by stab inoculation as described above. Plates were incubated at 37°C (without a plastic bag) for 24 hours before the diameter of the twitching motility zones were measured as above.

### III. Bacteriophage Sensitivity Assays.

Bacteriophage (phage) sensitivites were determined as described in Chapter 3.B.IV.

### IV. Negative Stain Preparations and Electron Microscopy.

For electron microscopy, bacteria were grown on TSB plates containing antibiotics as required, and a bacterial suspension was made by gently running a small amount of sterile phosphate buffered saline (PBS), pH 7.0, across the plate. Cells were adsorbed onto 3 mm, 200 mesh copper grids coated with Formvar and carbon. Bacteria were then stained with 2% (w/v) silicotungstic acid (STA), pH 6.9. Grids were examined using a Philips 201 transmission electron microscope. Images were recorded with Kodak fine grain positive release film. Technical assistance for electron microscopy was provided by Rakesh Bhatnagar (Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G 2E9).

### V. Isolation of Genomic DNA.

A single bacterial colony was used to inoculate 10 ml TSB and this culture was grown overnight at 37°C, 200 rpm, to stationary phase. Cells were pelleted by centrifugation, resuspended in 13.75 mM Tris-HCl, 0.75 mM EDTA, pH 8.0 containing 15% (w/v) sucrose, and incubated on ice for 10 minutes. Lysozyme was added to a final concentration of 0.5 mg/ml, incubated on ice for 10 minutes then 10% (w/v) SDS was added to a final concentration of 0.2% and incubated a further 10 minutes on ice. Lysed cells were then heated at 70°C for 5 minutes and an equal volume of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer) was added and the DNA sheared by passing the mixture through a 10 ml syringe fitted with 16 gauge needle. Two phenol extractions were performed using a half volume of TE saturated phenol, and the recovered DNA was subjected to an RNase digestion at room temperature for 1 hour by adding boiled 5 mg/ml RNase A to a final concentration of 0.1 mg/ml. Phenol extractions were carried out as above until the phenol:aqueous interface was clear. The chromosomal DNA was then precipitated by adding a 0.1X volume of 3 M sodium acetate, mixing gently, adding 2-3 volumes of cold (-20°C) 95% ethanol, and mixing by inversion until the DNA formed a large clump. This precipitated DNA was removed to another tube using a pasteur pipette and the ethanol allowed to drain off. It was then redissolved in 7 ml of TE buffer and precipitated again as above. The DNA was recovered by centrifugation for 10 minutes at 8000 x g, 4°C, the pellet was allowed to dry inverted at room temperature, and dissolved overnight at 4°C in 1 ml of sterile double distilled water. The DNA concentration was determined by reading the $A_{260}$ and $A_{280}$ in a Pye Unicam SP8-400 UV-Vis spectrophotometer (1.0 $A_{260}$ = 50 µg/ml DNA) (2).

### VI. Restriction Fragment Length Polymorphism Analysis and Southern Transfer of Chromosomal DNA.

Restriction fragment length polymorphism (RFLP) analysis was performed by digesting chromosomal DNA, typically 1-2 µg of DNA, with 20 U of Hind III or Pst I restriction enzyme (Boehringer-Mannheim GmbH) in appropriate manufacturer's buffer and with the addition of molecular biology grade bovine serum albumin (BSA) to 50 ng/µl. Digestions (volume = 20 µl) were for 18 to 24 hours at 37°C. After addition of 5 µl of DNA gel-loading buffer III (27), digests were loaded on a 0.7% (w/v) agarose gel buffered with 40 mM Tris-acetate, 1 mM EDTA pH 8.0 (TAE buffer) and electrophoresed in TAE buffer at 70 milliamperes (mA) constant current until the bromophenol blue (fastest) marker dye was about 2 cm from the bottom of the gel. The DNA was visualized by staining the gel for 10-15 minutes in 0.3 µg/ml ethidium bromide solution at room temperature, washing with tap water for an equal amount of time, and observing it on an ultraviolet (UV) transilluminator. Photographs were made with a Fotodyne camera (f = 105 mm) with red filter, using a shutter speed of 0.5 second and a 4.6 f-stop and Polaroid Type 667 film.

All incubation steps for preparing the agarose gel for Southern transfer were done at room temperature with gentle shaking. First the gel was rinsed in milliQ water (Millipore Corporation) then depurinated for 10 minutes in 0.26 M HCl, rinsed again and the DNA denatured in 0.2 M NaOH, 0.6 M NaCl for 45 minutes. It was rinsed again and finally neutralized for 45 minutes in 1 M Tris-HCl, 0.6 M NaCl, pH 7-7.5. The gel was placed face down on Whatman 3MM filter paper, cut to size, on top of sponges soaking in 1.5 M NaCl, 0.15 M $Na_3$ citrate (10X SSC). A Hybond-N nylon membrane (Amersham International plc) cut to size and marked for later orientation, was placed on top and smoothed out so that no air bubbles remained between it and the gel. This was overlayed with another piece of Whatman 3MM and a 3-4 inch thick layer of paper towels, all cut to size. A weight was placed on top of this assembly and the DNA allowed to transfer to the membrane by capillary action at room temperature overnight. After transfer was complete, the DNA was UV cross-linked to the membrane in a Bio Rad GS Gene Linker using the Southern Damp preset program (150 mJoules). The dry membrane was stored in the dark until probed.

The RFLP membrane was probed according to the instructions of the nylon membrane manufacturer. The membrane was placed in 0.9 M NaCl, 0.05 M $NaH_2PO_4$, 5 mM EDTA pH 7.7 (5X SSPE), 5X Denhardt's solution (27), 10% (w/v) SDS, then denatured, sonicated calf thymus DNA was added to a final concentration of 0.02 mg/ml and prehybridized at 65°C for 1 hour. Denatured, digoxygenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-dUTP)-labeled *pilA* gene probe was added and hybridization continued for at least 12 hours. The filter was washed twice for 10 minutes at room temperature with 2X SSPE, 0.1% (w/v) SDS, once at 65°C for 15 minutes with 1X SSPE, 0.1% (w/v) SDS, and once at 65°C for 10 minutes with 0.1X SSPE, 0.1% (w/v) SDS to ensure detection of identical or nearly identical sequences.

Visualization of hybridized bands was done by a chemiluminescent method with Boehringer Mannheim's Lumi Phos 530 (Lumi Phos) reagent using the manufacturer's protocol. After exposure to Lumi Phos, the membrane was sealed between acetate sheets and incubated at 37°C for 30 minutes to bring the light output to its maximum. The filter was exposed to Kodak X-OMAT X-ray film for 1 hour at room temperature and the film developed in a Fuji RG II X-ray Film Processor.

### VII. Bacterial Adherence Assays.

Adherence assays testing the ability of bacterial strains to bind to A549 human lung pneumocyte monolayers were performed as described in Chapter 3.B.III.

### VIII. Preparation and SDS-PAGE of Whole Cell Lysates.

Whole cell lysates were prepared by growing 10 ml cultures of PAO1, PAO4141, FA2, FA3, and BLP-3 in TCS + 5 mM $MgCl_2$ with antibiotics where appropriate. These were grown overnight at 37°C, 150 rpm. Cultures were dispensed into sterile 2 ml microfuge tubes (1.5 ml per tube), spun at top speed in an Eppendorf Centrifuge 5414 C for 5 minutes to pellet cells, and the supernatant discarded. Cells were resuspended in 100 µl protein loading buffer (120 mM Tris-HCl, pH 6.8, 3.8% (w/v) SDS, 3.8% (v/v) 2-

mercaptoethanol, 0.19% (w/v) bromophenol blue) and incubated in a boiling waterbath for 5 minutes to lyse the cells.

Samples (10 µl) were loaded in duplicate on a sodium dodecyl sulfate-polyacrylamide separating gel composed of 15% (w/v) polyacrylamide (15% SDS-PAG) with a 7% stacking gel (27). BRL pre-stained low molecular weight protein standards (5 µl) were included as was 2 µl of a 2.5 mg/ml PAK pilin solution in 0.01% sodium azide. Protein loading buffer was added to all samples (10 µl), standards (20 µl), and the pilin solution (20 µl), and all samples (except for standards) were incubated in a boiling waterbath for 20 seconds before loading. Samples were boiled for only 20 seconds to prevent heat-mediated aggregation of pilin subunits (39). The SDS-PAG was pre-run at 50 V constant voltage for 10 minutes to remove free acrylic acid from the gel, samples were loaded, and the gel was electrophoresed at 150 V in 1X electrode buffer (0.025 M Tris, 0.2 M glycine, 0.1% (w/v) SDS) until the dye front reached the separating gel. The voltage was then decreased to 40 V and the gel allowed to run overnight until the dye front was within 1-2 cm of the bottom of the separating gel.

Half of the electrophoresed gel was stained (all staining and destaining steps were carried out at room temperature with gentle mixing) in Coomassie Brilliant Blue R250 (5 mg/ml) in 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid for 40 minutes, and destained by soaking the gel in destain solution (10% (v/v) methanol, 10% (v/v) glacial acetic acid) for 8-24 hours with 2 or 3 changes of solution. A large (11.5" x 17") Scott Precision Wipe was also soaked with the gel during the destaining procedure to absorb the excess stain. The gel was then incubated in 5% (v/v) glycerol, 1% (v/v) glacial acetic acid for 8-24 hours and dried down according to manufacturer's instructions on a Hoefer Scientific Instruments Drygel Sr. Slab Gel Dryer Model SE 1160 at 80°C for 1 hour.

The proteins from the other half of the PAG were electrophoretically transferred to 0.45 micron nitrocellulose (Bio-Rad Trans-Blot Transfer Medium) by the Western Blotting method of Towbin et al (1979) (37). The gel was soaked in Western Tank Buffer (25 mM Tris, 200 mM glycine, 20% (v/v) methanol) while nitrocellulose and two pieces of Whatman 3MM were cut to the size of the gel then a "gel sandwich" was assembled as per manufacturer's instructions, taking care to remove any air bubbles between the gel and the nitrocellulose membrane. The "sandwich" was loaded in a water-cooled Bio-Rad Trans Blot Western Blotting Tank containing Western Tank Buffer at 4°C and the gel transferred onto the nitrocellulose for 10 minutes at 50 mA then 3-4 hours at 200 mA (all at 4°C with stirring of the buffer). When transfer was complete, if detection was not performed immediately, the damp membrane was heat-sealed in a Micro-Seal bag with a small amount of Western Tank Buffer and stored at 4°C.

## IX. Preparation of Pilin-Specific Polyclonal Antiserum and Immunodetection of Western Blots.

Non-piliated *P. aeruginosa* BLP-3 was grown from a single colony in 3 ml TSB for 6-8 hours at 37°C, 50 rpm on a tube roller and 1 ml of that was used to inoculate 100 ml of TSB which was grown for 16 hours at 37°C without shaking. Ice-cold bacterial cells were broken in the culture fluid by sonicating 4X 1 minute with a Branson Sonifier 450 (duty cycle = constant, output = 1, time = hold) fitted with the medium sized probe. 160 µl of rabbit anti-PAO antiserum (titer = $1.6 \times 10^6$) was added to the broken cells and the mixture incubated in a tightly closed sterile container for 48 hours at 4°C with gentle shaking to allow adsorption of antibodies specific for non-pilus PAO antigenic sites. The PAO pili-specific antibodies were recovered by centrifuging the suspension for 10 minutes at 5800 rpm, 4°C to pellet cell debris and the supernatant filtered through a 0.45 µm filter. The resultant preadsorbed anti-PAO pili-specific antiserum was stored frozen at -20°C.

Pilin bound to a nitrocellulose membrane was detected by a colorimetric method involving detection of the protein by primary antibody and detection of bound antibody by a secondary antibody-alkaline phosphatase (AP) conjugate. The procedure was carried out at room temperature, unless otherwise noted, with gentle mixing on a rocker platform. The nitrocellulose membrane was washed 5X for 2 minutes with Tris buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5-8.0) then blocked overnight at 4°C in TBS + 0.1% (v/v) Tween 20 + 3% (w/v) bovine serum albumin (TTBS-3% BSA). The membrane was then washed 2X for 10 minutes with TTBS-0.05% (w/v) BSA and incubated for 2 hours in a 1/1875 dilution of preadsorbed anti-PAO pili-specific antiserum. All antibody dilutions were done in TTBS-0.05% (w/v) BSA. Before addition of the secondary antibody, the membrane was washed 4X for 5 minutes with TTBS-0.05% (w/v) BSA to remove non-specifically bound primary antibody. A 1/2000 dilution of affinity isolated goat-anti-rabbit IgG-AP conjugate (titer = 1/8000) purchased from Sigma Immunochemicals was added to the membrane and incubated for 2 hours, then the membrane was washed 4X for 5 minutes with TTBS to remove non-specifically bound secondary antibody. The membrane was equilibrated for 5 minutes in 100mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ (AP buffer), and the colour reaction carried out by incubating it in freshly prepared AP substrate solution (0.1 ml of 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate-p-toluidinium salt

(BCIP) in dimethylformamide + 0.2 ml of 50 mg/ml nitro-blue tetrazolium (NBT) in dimethylformamide + 30 ml AP buffer) until the desired band intensity was achieved. The colour reaction was stopped by adding 50 ml TBS-20 mM EDTA (AP stop solution) to the membrane in AP substrate solution and incubating a further 2 minutes. Developed membranes were rinsed with milliQ water, allowed to air-dry in the dark, and heat sealed in a Micro-Seal bag for storage.

### X. Isolation and Characterization of Whole *P. aeruginosa* Membrane Fractions.

The method used to isolate whole membranes from *P. aeruginosa* was a modification of a membrane isolation method used by R. E. W. Hancock (14). A starter culture of the bacterial strain was grown from a single colony in 10 ml TSB (with antibiotics if required) for 6-8 hours at 37°C with gentle shaking. This was used to inoculate 1 litre of the same media and the bacteria were grown for 12 hours at 37°C, 100 rpm. The culture was blended for 2 minutes on low speed in a 4 litre Waring Blender to shear extracellular pili from the cells, and the cells recovered by centrifugation for 5 minutes at 9000 x g, 4°C. Approximately 300 ml of this supernatant was saved and pooled with the subsequent wash supernatants for determination of extracellular pili. Centrifuged cells were washed twice with 100 ml of ice-cold 30 mM Tris-HCl, pH 8.0 (Tris buffer). For the whole membrane preparation, all steps were carried out on ice unless otherwise noted.

The cell pellet was resuspended in 5 ml of 20% (w/v) sucrose in Tris buffer, 250 µl of 1 mg/ ml crude pancreatic DNase in Tris buffer and 12.5 µl of 20 mg/ml RNase A were added, and the suspension incubated at room temperature for 20-30 minutes, then subject to a freeze-thaw to enhance cell breakage. Bacteria were broken twice at 20,000 psi in a 4°C French Pressure Cell. Unbroken cells and cell debris were removed by centrifugation at 9000 x g, 4°C, for 10 minutes. The supernatant, containing the membranes, was saved ( 0.1 ml was removed for SDS-PAGE and protein determination) and loaded on top of a two step sucrose gradient consisting of 1 ml 70% (w/v) sucrose in Tris buffer and 6 ml 20% (w/v) sucrose in Tris buffer. The tubes were spun for at least 4 hours in the SW41Ti rotor at 39,000 rpm, 4°C in a Beckman L8-80M ultracentrifuge. The crude envelope fraction banded at the 20%-70% interface and was collected from above with a Pasteur pipette. The envelopes were washed 3X with 30 mM Tris-HCl, pH 8.0 containing 1 mM $MgCl_2$ ($Mg^{2+}$ helps maintain the integrity of the plasma membrane), and pelleted for 30 minutes at 47,000 rpm, 4°C in a 60Ti rotor. The membranes were treated with 100 µg/ml RNase A and 1 mg/ml egg white lysozyme, final concentrations, for 2-3 hours at 37°C and washed again 3X as above. The final pellet was resuspended in 1 ml of 30 mM Tris-HCl, pH 8.0 + 1 mM $MgCl_2$ and this preparation was called "whole membranes".

Whole membranes were characterized by SDS-PAGE, BCA protein assay and by assaying for succinate dehydrogenase and 2-keto-3-deoxyoctonic acid, markers for the inner and outer membrane, respectively. All samples were subject to SDS-PAGE on a 15% separating gel with 7% stacking gel. The cleared lysate resulting from cell breakage and centrifugation, and pellets from washes were prepared as 5 µl samples plus 20 µl protein loading buffer. 500 µl of the wash supernatants were dried down in a Savant SC110 speed vac and resuspended in 20 µl of protein loading buffer. All samples, except for molecular weight standards (BRL pre-stained low mw standards), were incubated in a boiling water bath for 3 minutes just prior to loading on the gel. Running, staining, and drying of the gel was done as described in section 5.B.VII.

Total protein in the whole membranes was determined using the BCA protein assay (Pierce Chemical Company). Manufacturer's instructions were followed except that the volumes of samples and reagents used for assays were halved. The enhanced assay protocol was used in which tubes were incubated at 60°C for 30 minutes to allow colour development. All tubes were read at an absorbance of 562 nm using a milliQ $H_2O$ blank and BSA standards ranging from 10 µg/ml to 250 µg/ml.

### XI. Shearing of Extracellular Pili and Detection of Pilin in Shear Fluids.

Pili were sheared from cells in a Waring Blender during the whole membrane isolation procedure as outlined in section 4.B.X. It was also noted that 400 ml of the shear fluid, out of a possible 1000 ml, was saved after bacteria were removed by centrifugation and that the two 100 ml washes to remove pili that were still stuck to the bacteria were pooled with the shear fluid. Pili were recovered from the shear fluid by the method of Paranchych et al (23). To 300 ml of the shear fluids, NaCl was added to 0.5 M, PEG 8000 was added to 1% (w/v), and these were stirred at 4°C for 24-48 hours to precipitate the pili. The pili were collected by centrifugation for 2 hours at 4°C, 44,500 rpm, in a 50.2Ti rotor, allowed to drain in inverted tubes at room temperature for 15 minutes, and resuspended in 0.2 ml of cold sterile milliQ $H_2O$. Pili recovered by this method were characterized by BCA protein assay, SDS-PAGE, and Western immunoblot as described in sections 5.B.VIII and 5.B.X.

### XII. Filtration and Detection of Extracellular Pilin.

A single colony was used to inoculate 2 ml of TSB + 5 mM $MgCl_2$ (plus antibiotics if required) and this starter culture was grown for 6-8 hours at 37°C, 50 rpm. 0.5 ml of this was used to inoculate 50 ml of TSB + 5 mM $MgCl_2$ (plus antibiotics if required) that was then grown as a standing culture for 16 hours at 37°C. At the beginning of the assay, cultures were diluted for plateouts to determine cell concentration and phage sensitivity tests were carried out as described in Chapter 3.B.IV. The bacterial cultures were filtered, 5 ml per filter, through Millipore filters of 0.05 µm pore size (cat # VMWP 047 00) into 1 litre filter flasks kept on ice. Pili were precipitated from the filtrates by the method described in section 5.B.XI above and characterized by Western immunoblot and by BCA protein assay.

### XIII. Cloning of a Mutant *pilG* Gene Using Polymerase Chain Reaction.

Primers for amplifying the *pilG* gene by polymerase chain reaction (PCR) were designed using the sequence of the gene published and deposited in the Gen-Bank data library (accession number L10831) by Darzins (8). The sequences of the primers were:

BC05: 5'-CCAAGGATCCCCATCACGAAAT-3' $T_m = 66^oC$
BC06: 5'-GCAAGAATTCCTCCGAAAGGGG-3' $T_m = 68^oC$

The primers were designed with 4 extra bases at their 5' ends to promote more efficient digestion of the PCR product by EcoRI (BC06) and BamHI (BC05) since the recognition sequences would be close to the ends of the DNA fragment. The melting temperatures ($T_m$) of the primers were obtained from Molecular Biology LabFax (1). The primers were ordered from a Biological Sciences Department DNA synthesis service and synthesized on an Applied Biosystems Inc. DNA synthesizer, model number 381A or 391.

Determination of the optimum conditions for PCR included using various amounts of digested chromosomal DNA as a template. All digestions were done with Boehringer Mannheim or New England Biolabs restriction enzymes according to the manufacturer's instructions. It was found that the best product/non-specific background ratio was obtained when 100-200 ng of PAO4141 chromosomal DNA cut with Pst I or 250-400 ng of FA2 chromosomal DNA cut with Pst I were used in the PCR reaction. The total number of PCR cycles run that minimized non-specific product without affecting desired product yield was 25.

The final conditions for the PCR reactions were:

| PCR Reaction Component | PAO4141 | FA2 |
|---|---|---|
| 10X Vent Polymerase Buffer | 10 µl | 10 µl |
| 1 mM dNTP mix | 10 µl | 10 µl |
| 50 pmol/µl BC05 | 2 µl | 2 µl |
| 50 pmol/µl BC06 | 2 µl | 2 µl |
| chromosomal DNA digested with Pst I | 200 ng | 400 ng |
| sterile milliQ water | to 99 µl | to 99 µl |
| 1 U/µl Vent polymerase (hot start) | 1 µl | 1 µl |

Reaction Times For MJ Research PTC-200

| | | |
|---|---|---|
| Denature | 95°C for 5 minutes | |
| Hot Start | 80°C for 30 seconds | |
| Anneal | 58°C for 2 minutes | |
| Extend | 72°C for 2 minutes | |
| Denature | 95°C for 1 minute | Repeat 23 X |
| Anneal | 58°C for 2 minutes | |
| Extend | 72°C for 2 minutes | |
| Extend | 72°C for 4 minutes | |
| | 4°C hold | |

Evaluation of the PCR product was carried out by adding 5 µl of DNA gel-loading buffer III to 10 µl of a finished reaction and loading the sample on a 1.5% (w/v) agarose gel buffered with 40 mM Tris-acetate, 1 mM EDTA pH 8.0 (TAE). DNA gel-loading buffer III (5 µl) was also added to 1 µg of DNA VII size standard (Boehringer Mannheim GmbH), loaded, and the gel electrophoresed in TAE buffer at 70 mA constant current until the bromophenol blue marker dye was 2 cm from the bottom of the gel. The DNA bands were visualized by staining with ethidium bromide and photographed as described previously in section 5.B.V. After agarose gel analysis of the reaction mixture, product from the remainder of a PCR reaction was purified according to manufacturer's instructions using a QIAquick Spin PCR Purification Kit manufactured by QIAGEN GmbH. Briefly, a DNA-binding resin was added to the remaining 90 µl of PCR reaction mixture, loaded onto a spin column, and the bound DNA washed with buffer containing ethanol. The bound DNA was then dried and eluted with 50 µl TE buffer, pH 8.0.

Purified products from the PAO4141 and FA2 PCR reactions and the vector pBluescript SK+ were digested with Eco RI and Bam HI in Boehringer Mannheim's Buffer H according to the manufacturer's instructions. Each reaction was extracted twice with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol saturated with TE buffer, and the organic phases back extracted with sterile milliQ $H_2O$. Aqueous phases were pooled and extracted twice with an equal volume of water-saturated ether to remove any residual phenol. After residual ether had been evaporated, a 0.1 volume of 3 M sodium acetate (NaOAc) pH 5.2 was added, mixed, then the DNA precipitated by the addition of 2.5 volumes of cold 95% ethanol and incubation of the tubes in dry ice for at least 30 minutes. The precipitated DNA was recovered by centrifuging the tubes at 12,000 rpm, $4^oC$ for 30 minutes. The pellets were washed with cold 70% ethanol, centrifuged as before, evaporated to dryness, and redissolved in 10 μl of sterile milliQ $H_2O$. Each purified digest (1 μl) was then run on a 1.5% agarose gel to approximate concentrations of the fragments.

Ligation reactions were set up as follows with the Eco RI, Bam HI-digested fragments and Bluescript vector:

1. 25 ng FA2 PCR product + 40 ng pBluescript SK+
2. 50 ng PAO4141 PCR product + 40 ng pBluescript SK+
3. 20 ng pBluescript SK+ with no insert DNA

The ligations were carried out in Boehringer Mannheim T4 ligase buffer with 0.5 U of T4 DNA ligase per reaction (10.5 μl total volume). Incubations were performed at $15^oC$ in the PTC-200 DNA Engine for 48 hours. 5 μl of each ligation mixture was run on a 1.5% agarose gel to check for ligation products, then 3 μl of each ligation reaction was transformed into *E. coli* DH5α by the Hanahan method (13, 27). The transformation mixture was grown at $37^oC$ for 1 hour then 1/5 and 1/25 dilutions plated on TSB agar containing 100 μg/ml ampicillin, 0.16 mg/ml isopropyl β-D-thiogalactopyranoside (IPTG), and 0.13 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Plateouts of the entire ligation mixtures yielded 37 FA2 transformants (15 with inserts), and 13 PAO4141 transformants (6 with inserts). Plasmid DNA from each of the transformants was prepared by the boiling method of Holmes and Quigley (18, 27). RNase A was then added to each preparation to a final concentration of 20 μg/ml, and the tubes incubated at room temperature for 10 minutes. 10 mM Tris-HCl, 1 mM EDTA (TE buffer), pH 8.0 was added to each tube to bring the volume up to 100 μl, followed by 50 μl of 30% (w/v) PEG 8000, 1.5 M NaCl, mixing, and an incubation on ice for 30 minutes. DNA was pelleted by centrifuging the tubes at 12,000 rpm, $4^oC$, for 10 minutes, and washed 3X with cold 70% ethanol. After the final wash, the pellets were dried in a Savant SC110 Speed Vac and redissolved in 25 μl of TE buffer, pH 8.0, each.

Characterization of six of each of the PAO4141 and FA2 transformants was carried out in two ways:

1. The plasmids were digested with Pvu II which cut on either side of the multiple cloning site of pBluescript SK+ and not within the *pilG* fragment and the inserts checked for appropriate size (0.7 kb) on a 0.7% agarose gel.
2. The plasmids were digested with Sal I which cut once in the *pilG* fragment and once in the pBluescript SK+ vector and the fragments checked for appropriate size on a 1.5% agarose gel.

### XIV. Sequencing of *pilG* Genes.

Automated sequencing of the cloned *pilG* genes was carried out on an ABI Model 373A DNA Sequencer. Plasmid DNA for the sequencing reactions was prepared using the Wizard 373 DNA Purification System (Promega Corp.), which involved binding plasmid DNA to a resin, washing, eluting, and concentrating the DNA. The DNA solutions were made up to 20 µl with sterile milliQ $H_2O$ and diluted 1/10 (50 µl total volume) in sterile milliQ $H_2O$ to read the absorbance at 260 nm. Absorbances were read in an Ultrospec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech Ltd) using 50 µl cuvettes and the concentration of double stranded (ds) plasmid DNA calculated using the formula:

$$\mu g/ml\ DNA = Dilution\ Factor\ (50\ \mu g\ dsDNA/A_{260})\ (A_{260}).$$

For sequencing, plasmid solutions were diluted to contain 1 µg of ds plasmid DNA in 5 µl of solution. Sequencing was done from the T3 and T7 universal primer sites on the pBluescript SK+ vector so that each clone was sequenced from each end of the *pilG* fragment such that the sequences overlapped to cover the entire 705 base-pair fragment. Three PAO4141 *pilG* clones and three FA2 *pilG* clones were sequenced in order to confirm that any variation from the published sequence was due to mutation and not due to incorrect copying of the template in the PCR reaction.

## C. Results

### I. Motility and Phage Sensitivity Profiles of *pilG* Mutants.

The motility and twitching motility of each mutant and its parent strain (FA2, PAO1; FA3, PAO4141) was confirmed before beginning the investigation. Stab inoculation of each of the bacterial strains onto LB agar plates (0.3% agar for flagellar motility, 1% agar for twitching motility) gave rise to radial zones of bacterial growth that could be measured. Table 4.2 shows the diameters of flagellar and twitching motility zones.

Table 4.2. Flagellar motility, twitching motility and phage sensitivity patterns.

| Bacterial strain | Flagellar motility diameters (mm) | Twitching motility diameters (mm) | Phage sensitivity [a] |
|---|---|---|---|
| PAO1 | 32.0 | 19.5 | $B9^s$ $F116^s$ $Pf1^r$ $PO4^s$ |
| PAO4141 | 30.5 | 12.0 | $B9^s$ $F116^s$ $Pf1^r$ $PO4^s$ |
| FA2 | 31.0 | 0 | $B9^s$ $F116^s$ $Pf1^r$ $PO4^s$ |
| FA3 | 27.0 | 0 | $B9^r$ $F116^r$ $Pf1^r$ $PO4^r$ |

[a] sensitive, s; resistant, r.

All strains were found to express flagella and this was confirmed for each strain by observing a wet mount by phase contrast microscopy. All strains were also observed to be very actively motile by this method.

Phage sensitivity profiles were determined for each strain by spotting phage onto bacteria on TSB agar and growing the plate until a lawn of bacteria had formed. Resistance to the phages was observed as normal bacterial growth over areas where phages had been applied and sensitivity was observed as a clear zone with no bacterial growth over areas where the phages had been spotted. The two background strains, PAO1 and PAO4141 had typical PAO1-type phage sensitivity patterns as seen in table 4.2. They were sensitive to all the pilus-specific phage except for Pf1 which is a filamentous phage that binds specifically at the tip of *P. aeruginosa* PAK pili but does not bind to PAO1-type pili. This indicated that PAO1 and PAO4141 were expressing functional (retractable) pili. The *pilG* mutant FA2 also gave a typical PAO1-type phage sensitivity profile (see table 4.2) which was somewhat unexpected since FA2 was reported to be non-piliated. Darzins suggested that the FA2 mutant perhaps had a tip of the pilus protruding through the outer membrane and, therefore, could be infected by some of the pilus-specific phages (8). It is possible that this is the case and that three of the four phages tested in this study were able to bind to the protruding pilus tip and infect the bacterial cells. Previous electron microscopic studies showed that phage Pf1 bound to the tip of PAK pili and that phages B9, F116 and $PO_4$ bound to the sides of PAO1 and PAK pili (W. Paranchych, personal communication). The phage sensitivity profile of the FA3 *pilG* insertion mutant was identical to other non-piliated mutants (data not shown) in that it was resistant to all of the phages. This supports Darzins' observation that this mutant did not appear to be piliated (8).

## II. Transmission Electron Microscopic Studies of *pilG* Mutants.

For transmission electron microscopy (TEM), bacterial cell suspensions were negatively stained with 2% (w/v) STA, pH 6.9 and observed in a Philips 201 Transmission Electron Microscope. Flagella are the thick filaments seen in Fig. 4.5 for PAO1, PAO4141, FA2

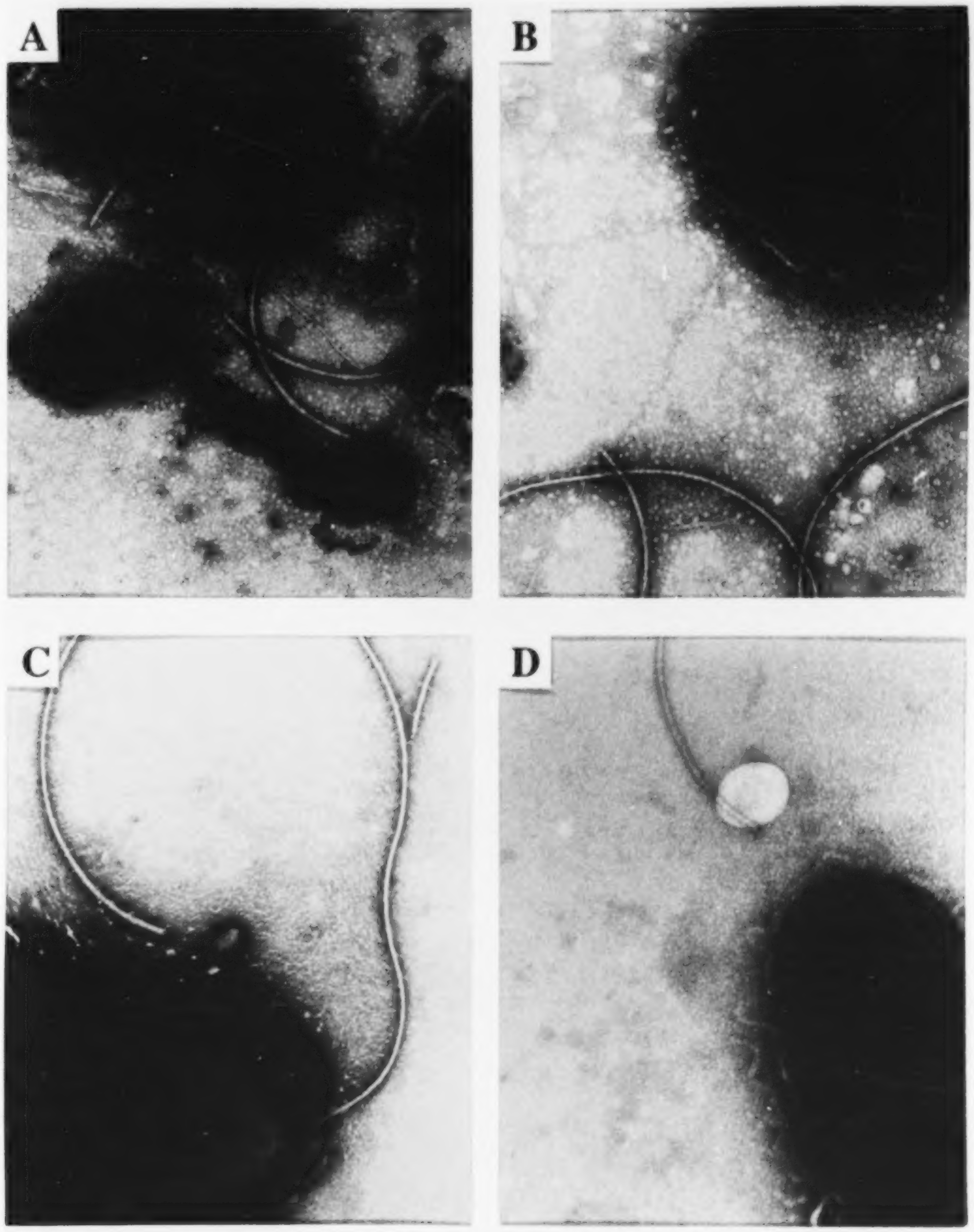


Fig. 4.5. Piliation of wild-type and mutant *P. aeruginosa* strains. PAO1 (**A**), PAO4141 (**B**), FA2 (**C**), and FA3 (**D**) were negatively stained with 2% (w/v) silicotungstic acid (pH 6.9) and observed by transmission electron microscopy. Polar pili were observed in PAO1 and PAO4141 (wild-type) preparations but not in preparations of the pilG mutants FA2 and FA3. Magnification is 47,300X.

and FA3, which agrees with the results of the motility studies. The fact that the flagella were usually not attached to the cells is consistent with prior observations that flagella were normally sheared from the cells during sample preparation for TEM. The thin filaments at the poles of PAO1 and PAO4141 are pili and unattached pili are also visible. PAO1 is usually observed with 6-8 pili per cell (W. Paranchych, personal communication) and PAO4141 was observed to have from 3-7 pili at a pole of the cell, comparable to the level of piliation for PAO1. No pili were observed in FA3 preparations, supporting the phage sensitivity results that suggested it was non-piliated. FA2 appeared to be non-piliated except for two bacteria in an FA2 preparation that were observed to have one polar pilus each. This suggests a possible "leaky" phenotype that may allow occasional pilus assembly. At the level of resolution afforded by the TEM, it was not possible to determine if FA2 had any "pre-pilus" structures at its cell surface. For this reason, it cannot be said that the TEM results for FA2 agreed or disagreed with the phage sensitivity profile, although, certainly, FA2 is not piliated in the same manner as its wild-type parent, PAO1.

### III. Detection of the Pilin Structural Gene and Protein Subunit by Southern Hybridization and Western Immunoblotting.

Because the suggestion of the phage sensitivity, twitching motility and TEM data was that the piliation of the *pilG* mutants FA2 and FA3 was different from that of wild-type, detection of the structural pilin gene, *pilA*, was carried out by Southern hybridization in order to determine that the structural gene itself was not affected in these mutants. Chromosomal DNA was isolated from each of the mutant and parent strains, digested with Hind III or Pst I, electrophoresed on a 0.7% (w/v) agarose gel, and the DNA transferred to a nylon membrane. The membrane was probed with a 1.2 kb DNA fragment containing the *pilA* gene cloned from *P. aeruginosa* strain K (PAK) (24). Figure 4.6 shows that PAO1, PAO4141, FA2 and FA3 all have the same RFLP pattern, that is, the *pilA* gene is on the same sized fragment for each digestion. Digestion of the chromosome with Hind III gives rise to a 1.2 kb *pilA*-containing band and digestion with Pst I gives rise to a 4.0 kb *pilA*-containing band. This pattern is typical of a PAO1-type *P. aeruginosa* and suggests that the *pilA* gene in the mutants is likely unchanged from the wild-type gene, agreeing with the complementation results obtained by Darzins for the FA2 and FA3 mutants (8).

### IV. Assessment of the Relative Virulence of *pilG* Mutants by Adherence to A549 Human Lung Pneumocytes and Virulence in a Mouse Model.

The most obvious question concerning the differences in piliation between the wild type parent strains and the *pilG* mutants was about how these differences affected the ability of

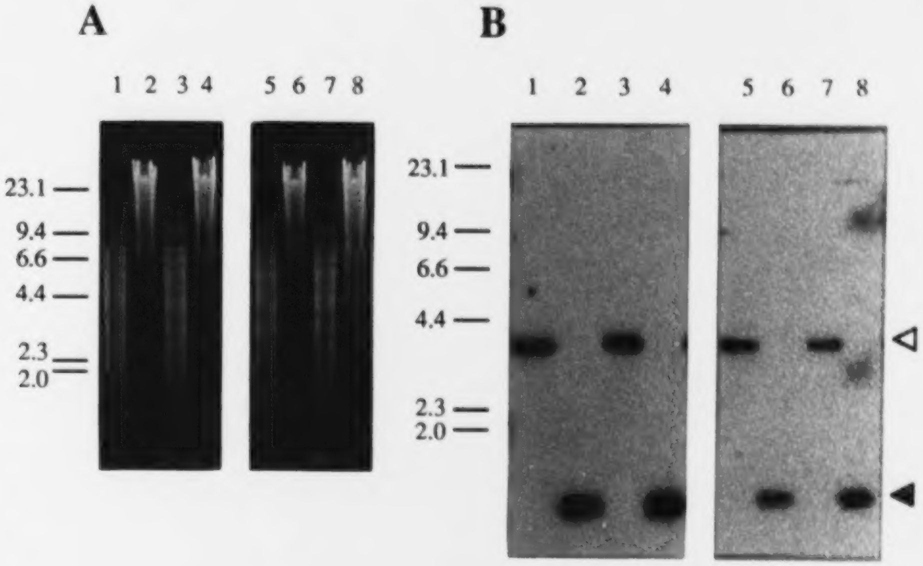


Fig. 4.6. Restriction Fragment Length Polymorphism (RFLP) analysis of *P. aeruginosa* strains. Chromosomal DNA (1.5 μg) from PAO1, PAO4141, FA2, and FA3 was digested with Pst I or Hind III, separated on a 0.7% (w/v) agarose gel, blotted onto nylon membrane, and probed with a DIG-dUTP-labeled 1.2 kb fragment from *P. aeruginosa* PAK containing the *pilA* gene sequence. The ethidium bromide-stained chromosomal digests are shown in (**A**) and a 60 minute, room temperature exposure of the probed membrane is shown in (**B**). Molecular weight standards (kb) are shown to the left. Lanes: (1), PAO1/Pst I; (2), PAO1/Hind III; (3), FA2/Pst I; (4), FA2/Hind III; (5), PAO4141/Pst I; (6), PAO4141/Hind III; (7), FA3/Pst I; (8), FA3/Hind III. The Pst I, Hind III banding pattern observed for all strains in (**B**) is typical of PAO1 *pilA* gene location on the chromosome: *pilA* occurs on a 1.2 kb Hind III fragment (filled arrowhead) and a 4.0 kb Pst I fragment (open arrowhead).

the bacteria to adhere to eukaryotic monolayers in a binding assay. Each strain was assayed for its ability to adhere to A549 human lung pneumocyte monolayers according to the standard adherence assay described in chapter 3. Briefly, bacteria were grown as a standing overnight culture at 37°C, washed, resuspended at a known concentration and various dilutions of that applied to A549 cell monolayers of known cell density. The bacteria were allowed to adhere for one hour with gentle mixing, then the monolayers were washed to remove non-adherent bacteria, trypsinized, and plated out to determine numbers of adherent bacteria. Figures 4.7, 4.8, 4.9 and 4.10 show the results of A549 pneumocyte adherence assays. The abilities of the two parent strains to adhere to A549 monolayers were quite different as PAO4141 demonstrated almost 5-fold fewer receptors than PAO1. This apparent lack of virulence was supported by the $LD_{50}$ results obtained from assays in susceptible A.BY/SnJ mice (Table 4.3). Of the two parent strains, PAO1 was far more virulent as shown by an $LD_{50}$ more than 100X lower than PAO4141, in addition to its greater ability to adhere to cultured human lung pneumocytes. The $LD_{50}$ for PAO4141 was so high that an $LD_{50}$ for FA3 was not done since, if anything, it was expected to be higher yet due to the *pilG* mutation. The adherence of FA2 to A549 cells was nearly identical to PAO1 with respect to the maximum number of receptors per A549 cell ($R_T$) and the dissociation constant ($K_D$) also approached that of PAO1, indicating that even though FA2 was not expressing normal pili, it was able to adhere at a wild type level. Table 4.3 shows that the $LD_{50}$ obtained for FA2 was higher than that for PAO1, but only by a small amount (a 1.2X difference) that was found not to be significantly different at 95% confidence limits by the T-test for significant difference of means. Compared with results obtained for PAO-MP, whose mutant pilins did not express the C-terminal disulfide loop (Chapter 3.C.IV-VI), the adherence and $LD_{50}$ data for FA2 suggested that, unlike PAO-MP, FA2 retained some form of adherence domain at the cell surface. This supports Darzins' suggestion that FA2 may be able to assemble a pre-pilus structure rather than a fully functional pilus (8).

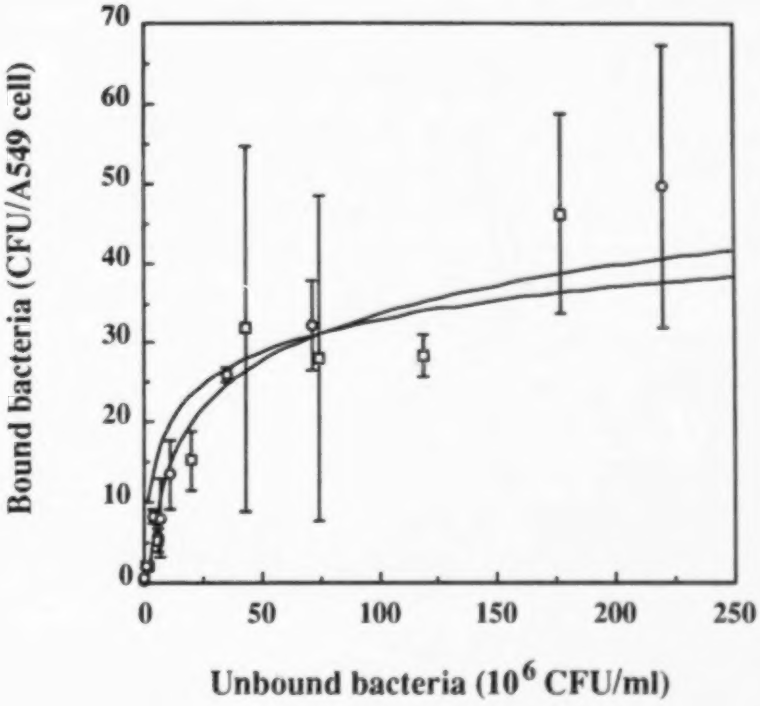


Fig. 4.7. Saturation curve of *P. aeruginosa* PAO1 (☐) and FA2 (O) adherence to A549 human lung pneumocytes. Increasing concentrations of each strain were incubated with approximately $10^5$ A549 cells per well for one hour. The monolayers were then washed, trypsinized, and assayed for adherent bacteria as described in Chapter 3.B.III. Data were plotted using Cricket Graph v1.3.2. Vertical bars indicate standard error.

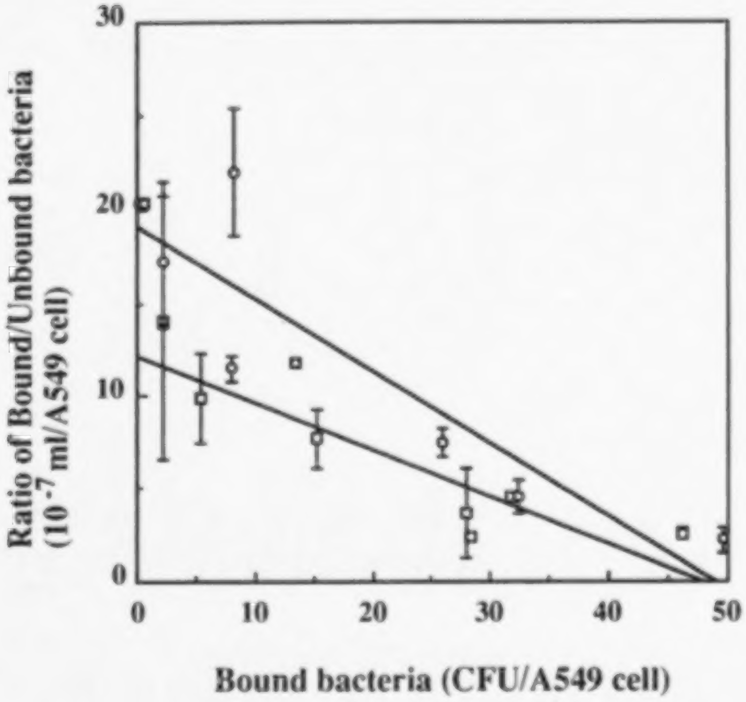


Fig. 4.8. Scatchard plot analysis of *P. aeruginosa* PAO1 and FA2 adherence data shown in Fig. 4.7. *P. aeruginosa* PAO1 (☐, $r^2$=0.822) and FA2 (O, $r^2$=0.818) adherence to A549 human lung pneumocytes was analyzed by the method of least-squares regression to calculate the total receptors per A549 pneumocyte and the dissociation constant for each strain. Data was plotted using Cricket Graph v1.3.2. Vertical bars indicate standard error.

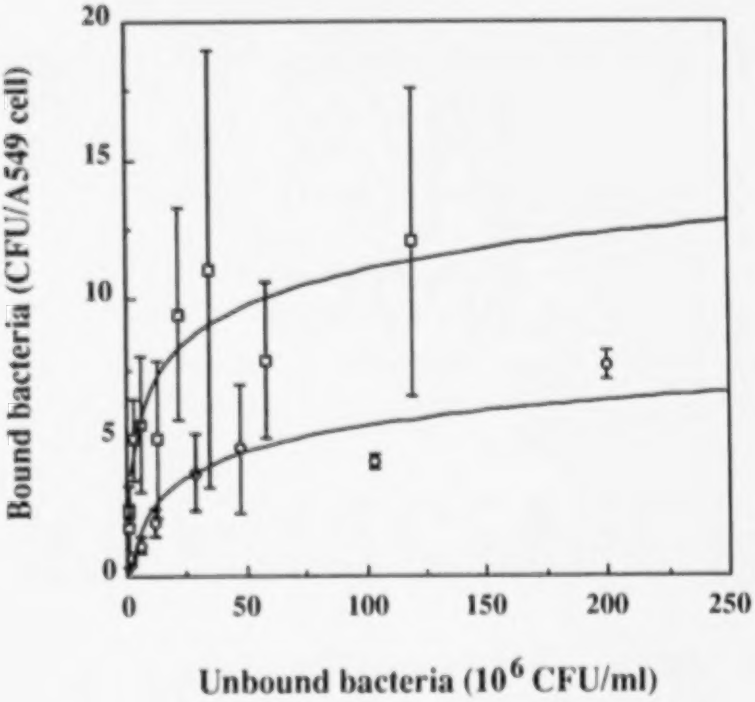


Fig. 4.9. Saturation curve of *P. aeruginosa* PAO4141 (□) and FA3 (O) adherence to A549 human lung pneumocytes. Increasing concentration of each strain were incubated with approximately $10^5$ A549 cells per well for one hour. The monolayers were then washed, trypsinized, and assayed for adherent bacteria as described in Chapter 3.B.III. Data was plotted using Cricket Graph v1.3.2. Vertical bars indicate standard error.

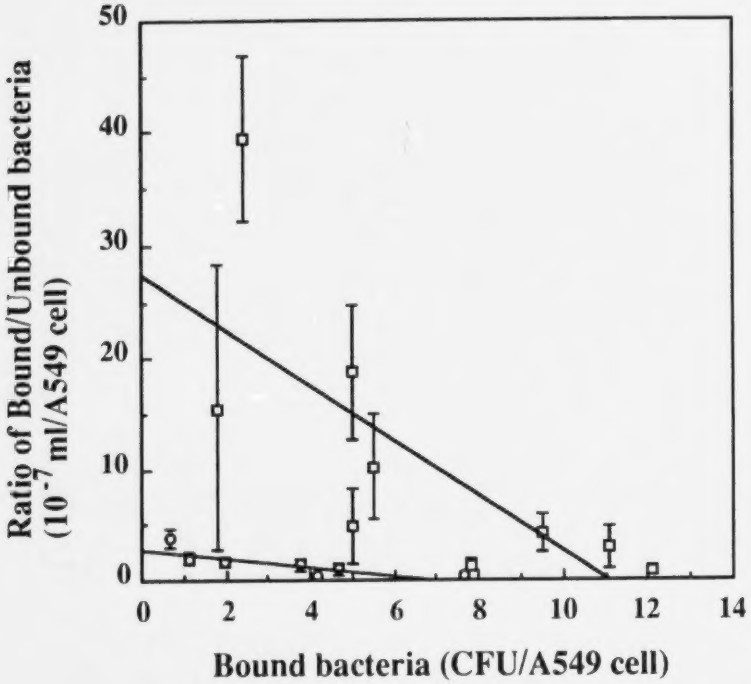


Fig. 4.10. Scatchard plot analysis of *P. aeruginosa* PAO4141 and FA3 adherence data shown in Fig. 4.9. *P. aeruginosa* PAO4141 (□, $r^2$=0.535) and FA3 (O, $r^2$=0.663) adherence to A549 human lung pneumocytes was analyzed by the method of least-squares regression to calculate the total receptors per A549 pneumocyte and the dissociation constant for each strain. Data was plotted using Cricket Graph v1.3.2. Vertical bars indicate standard error.

Table 4.3. Virulence characteristics of *P. aeruginosa* strains.

| Strain | $R_T$[a, b] | $K_D$[a] (CFU/ml) | $LD_{50}$[a, c] | Standard error[c] | # assays[d] |
|---|---|---|---|---|---|
| PAO1 | 48 | $4.0 \times 10^7$ | $7.0 \times 10^4$ | $\pm 2.5 \times 10^4$ | 17 |
| PAO4141 | 11 | $4.0 \times 10^6$ | $1.0 \times 10^7$ | $\pm 3.3 \times 10^6$ | 9 |
| FA2 | 49 | $2.6 \times 10^7$ | $8.1 \times 10^4$ | $\pm 1.3 \times 10^4$ | 8 |
| FA3 | 7 | $2.6 \times 10^7$ | n.d.[a] | n.d.[a] | n.d.[a] |

[a] Abbreviations: $R_T$, total receptors; $K_D$, dissociation constant; $LD_{50}$, 50% lethal dose; n.d., not determined.

[b] $R_T$ units are total receptors/A549 cell.

[c] $LD_{50}$ and standard error were calculated using the probit method (38). Units are CFU/mouse.

[d] Replicate assays included 8 or 10 mice/assay.

The level of adherence of FA3 to A549 pneumocytes compared to PAO4141 was similar but with fewer receptors per A549 cell (7 vs. 11). There was also approximately a six-fold difference in the $K_D$'s for FA3 and PAO4141, with FA3's being higher. This suggests that the disruption of the *pilG* gene in FA3 reduced its ability to adhere to A549 cells to a level lower than that of its parent strain, PAO4141. The FA3 mutant's inability to assemble pili led to a reduced ability to adhere to A549 pneumocytes in a binding assay.

### V. Determination of Cellular Distribution of Pilin.

When it had been determined that the two *pilG* mutants seemed to have different abilities to adhere to cultured A549 human lung pneumocytes, it was decided that the next step would be to determine the cellular distribution of the pilin protein in both wild type and mutant strains to confirm that the mutations in the *pilG* gene affected only the assembly of pilin monomers into a functional pilus, not the production of pilin. This was accomplished first by confirming the expression of pilin in whole cell lysates, next by detection of pilin in whole membrane isolates and comparing this to pili or pilin that could be mechanically sheared from the cell surface, and finally by addressing the question of whether or not the pilin subunits were excreted into the culture fluid if assembly into functional pili did not occur in the *pilG* mutants.

Whole cell lysates of PAO1, PAO4141, FA2, FA3, and BLP-3 (a Tn*501* insertion mutant in the structural *pilA* gene) were prepared and 10 µl of each was run in duplicate on a 15% SDS-PAG with BRL pre-stained low molecular weight standards and 1.7 µg of purified PAK pili. Fig. 4.11.A shows a crowded profile of the many proteins expected in a whole cell lysate. A Western immunoblot was performed on the duplicate of this gel and the pilin structural protein was detected by whole serum raised against *P. aeruginosa* PAO1 that had

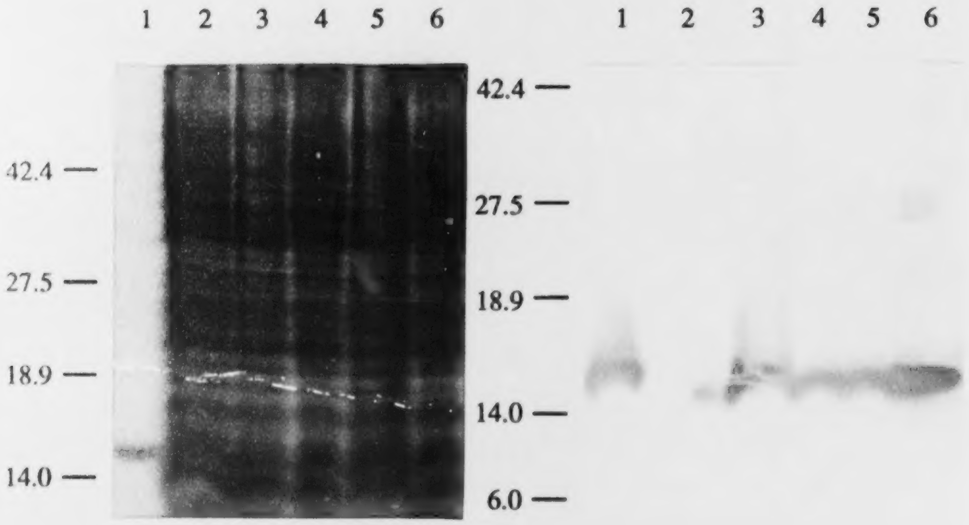


Fig. 4.11. Detection of pilin in whole cell lysates of P. aeruginosa strains. Whole cell lysates (10 µl) of PAO1, PAO4141, FA2, and FA3 were separated on a 15% (w/v) SDS-PAG (**A**). BLP3 was included as a negative control strain that did not produce pilin and 1.7 µg of purified PAK pilin was included as a positive control for pilin detection. Lanes: (1), purified pilin; (2), BLP3 whole cell lysate; (3), PAO1 whole cell lysate; (4), PAO4141 whole cell lysate; (5), FA2 whole cell lysate; (6), FA3 whole cell lysate. The proteins were electrophoretically transferred to a nitrocellulose membrane and pilin bands detected with pili-specific preadsorbed whole serum against PAO1 (**B**). Lanes: (1), PAO1 whole cell lysate; (2), BLP3 whole cell lysate; (3), PAO4141 whole cell lysate; (4), FA2 whole cell lysate; (5), FA3 whole cell lysate; (6), purified pilin. Molecular weight markers (kDa) shown to the left.

been preadsorbed to remove all antibodies except those directed against pili. Fig. 4.11.B shows that BLP-3, the negative control, clearly does not produce the pilin structural protein, PilA, as no band was detected in the whole cell lysate. This also indicated that the antiserum used was indeed specific for pili as all antibodies to other PAO1 antigens had been preadsorbed. For all other strains, including the *pilG* mutants, a single band of molecular weight 15.0 kDa reacted with the pilus-specific antiserum that corresponded to the major band detected in the purified PAK pili lane. The higher molecular weight band detected in this lane has previously been reported to be pilin dimer (41). These results indicate that the two *pilG* mutants, FA2 and FA3, are producing the pilin monomer and that the mutation in the *pilG* gene has not affected expression of the PilA protein.

This result led to the question of where the pilin was located in the mutant bacterial cells. If the *pilG* gene functioned in pili expression, it would be interesting to note whether or not the PilA subunit was being properly processed in the mutants, that is, did the mutant or absent PilG protein (FA2 and FA3 respectively) allow the pilin monomers to pool normally in the inner membrane (IM) and outer membrane (OM) of the bacterial cell as Watts et al (41) had previously shown? To address this question, any pili present were sheared from the FA2 and FA3 mutants and their parent strains by blending, whole membranes were then isolated from the cells, run in duplicate on a 15% SDS-PAG, and subject to Western immunoblot with detection again by the pili-specific preadsorbed whole serum raised against PAO1. Fig. 4.12.A shows the Coomassie blue stained gel. The gel shows the numerous faint bands typical of the IM and also the "major bands" (eg. 18 kDa, 23 kDa and 40 kDa) typical of the OM (14).

Fig. 4.12.B shows the results of a Western immunoblot carried out on the whole membrane preparations. 25 µg total protein was loaded for each sample along with 1.7 µg purified PAK pili. The PilA protein was detected with pili-specific preadsorbed whole serum directed against PAO1 and since any pili were sheared from the cells before the membrane isolation, the pilin detected was from membrane pools only (Watts et al (41) showed that even without blending, the centrifugation steps, including the 2-step sucrose spin, removed 99.96% of pili). Again, all strains show the presence of pilin in the whole membranes, indicating that the pilin protein is being processed and is probably being inserted into at least the inner membrane, and possibly the outer membrane as well. Since the band intensities are approximately equal for the *pilG* mutants and for their wild-type parents, it would appear that in the *pilG* mutants, the pilin is being expressed and processed

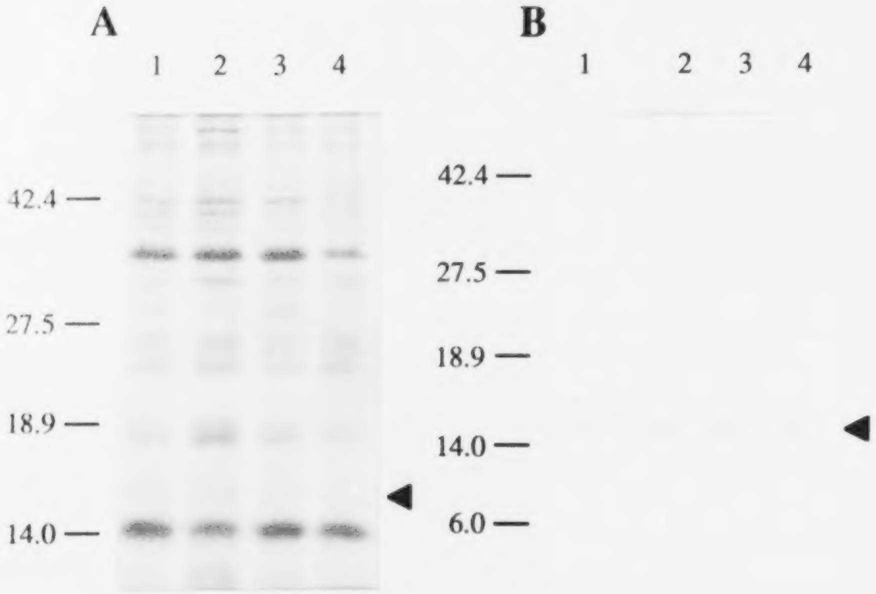


Fig. 4.12. Detection of pilin in whole membrane preparations from P. aeruginosa strains. Whole membrane preparations (25 µg total protein) of PAO1, PAO4141, FA2, and FA3, and 1.7 µg of PAK pilin were separated on a 15% (w/v) SDS-PAG (**A**). The proteins were electrophoretically transferred to a nitrocellulose membrane and pilin bands detected with pili-specific preadsorbed whole serum against PAO1 (**B**). Lanes: (1), PAO1 whole membranes; (2), PAO4141 whole membranes; (3), FA2 whole membranes; (4), FA3 whole membranes. Molecular weight markers (kDa) are shown to the left. Arrowheads indicate position of pilin bands.

as in the parent strains. This leads to the conclusion that the error in the *pilG* mutants is in the actual assembly of the pilin subunits into a functional pilus.

To further characterize the phenotype of the *pilG* mutants, two other experiments were done. The first was to attempt to isolate pili from the shear fluids created during the whole membrane isolation procedure in order to determine whether pilin would be detected in shear fluids from only the piliated strains. The second experiment involved filtering liquid cultures of the mutant and parent strains in order to determine if the *pilG* mutations caused the bacteria to excrete pilin subunits into the medium without polymerizing them into a pilus or if the pilin subunits stayed in the membrane as though blocked at a step just prior to polymerization of the subunits.

Shearing of the pili from the bacterial cells was accomplished by blending a liquid culture in a Waring blender for 2 minutes, centrifuging the cells and then washing them twice in 30 mM Tris-HCl, pH 8.0 (Tris buffer) to remove any pili sticking to the surface of the cells. The pooled blended culture supernatant and washes were referred to as shear fluids. Pili were purified from the shear fluids by precipitation with NaCl and PEG 8000. Equal amounts of total protein from the precipitates were run in duplicate on a 15% SDS-PAG with half of the gel stained with Coomassie blue and half subject to Western immunoblot analysis using pili-specific preadsorbed whole serum raised against PAO1 for detection of pilin. Fig. 4.13.A and Fig. 4.13.B show the Coomassie-stained gel and the Western immunoblot, respectively. The Coomassie-stained gel shows bands of far less intensity than the whole cell lysates. The pili-specific antiserum reacted with proteins from both the *pilG* mutants and the parent strains, indicating that pili or pilin was present in the shear fluids of all four strains. In the case of the piliated PAO1 and PAO4141 strains, the indication is that the pili are indeed being sheared from the cells by the blender treatment. In the case of the *pilG* mutants, the improperly functioning or missing PilG protein probably results in either excretion of pilin subunits or pili into the culture fluid.

To differentiate between excreted pilin subunits or the larger pilus structure, PAO1, PAO4141, FA2, FA3, and DB2, a hyperpiliated mutant incapable of pilus retraction, were grown in liquid culture, then filtered through 0.05 μm filters. Since intact pili are approximately 6 nm in diameter and 2.5 μm average length (12), it was reasoned that a filter with a 0.05 μm pore size would retain free pili and bacterial cells with attached pili, but allow short pieces of pilus and individual pilin subunits (15 kDa molecular weight) to pass through the filter. The filtrates were collected and any pili present were precipitated by

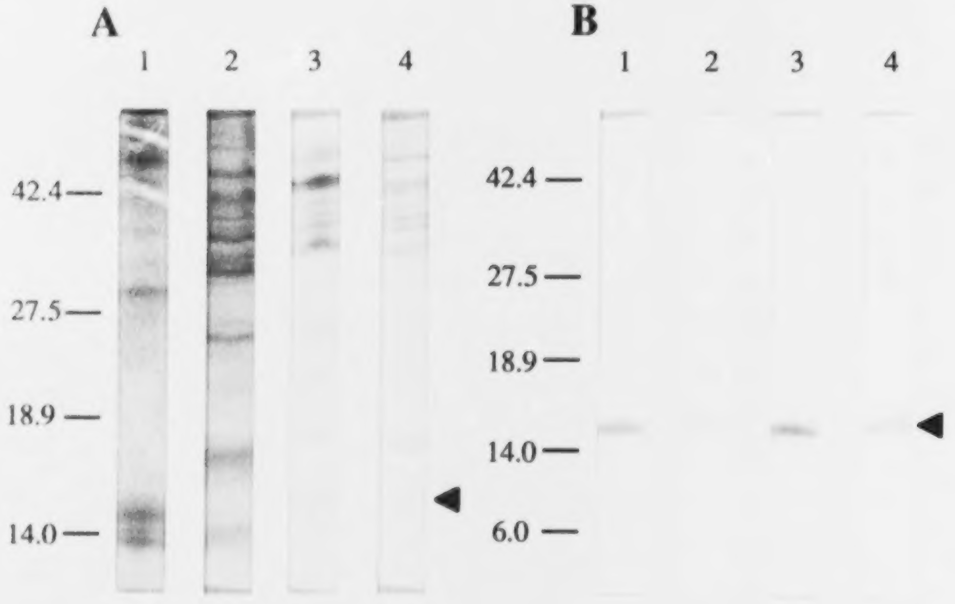


Fig. 4.13. Detection of pilin in shear fluids from P. aeruginosa strains. Shear fluid preparations (50 µg total protein, except for PAO1) from PAO1, PAO4141, FA2, and FA3 were separated on a 15% (w/v) SDS-PAG and stained with Coomassie Blue (A). The proteins from duplicate wells were electrophoretically transferred to a nitrocellulose membrane and pilin bound to the nitrocellulose was detected with pili-specific preadsorbed whole serum against PAO1 (B). Lanes: (1), PAO1 shear fluid; (2), PAO4141 shear; (3), FA2 shear; (4), FA3 shear. Molecular weight standards (kDa) are shown to the left. Arrowheads indicate the positions of pilin bands.

the addition of NaCl and PEG 8000 as above. 50 μg of the total protein recovered was run in duplicate on a 15% SDS-PAG. Half of the gel was stained with Coomassie blue and half subject to Western immunoblot analysis using pili-specific preadsorbed whole serum raised against PAO1 for detection of pilin. Fig. 4.14.A shows the Coomassie-stained gel and Fig. 4.14.B shows the Western immunoblot. Very faint bands corresponding to the PAK pilin band were detected from all strains indicating that in all cases, a small amount of pilin, or in the case of the piliated organisms, possibly also short sections of pili, were present in the culture medium; note that this assay could not distinguish between pilus fragments and free pilin monomers or dimers in solution. That the band for the hyperpiliated DB2 mutant is more intense than the other bands supports the idea of detecting small sections of pili that have broken off of the pilus because mutants with non-retractable pili have been found to result in cells with more pili (hyperpiliated) and also pili of longer average length than wild-type cells (3, 6). This suggests that perhaps the likelihood of pili breakage is greater with hyperpiliated cells, resulting in the greater amount of extracellular pilin detected for DB2. Taken together, these results suggest that with both of the *pilG* mutants, pilin subunits are processed normally to form pilin pools in the membranes but they are not polymerized into a pilus structure. It also seems probable that bacteria producing pilin, whether it is assembled or not, will export a small amount of the pilin and that piliated bacteria are subject to mechanical breakage of their pili.

### VI. Cloning and Sequencing of the FA2 *pilG* Gene.

In order to understand the nature of the mutation in the FA2 *pilG* gene, the mutant gene from the FA2 chromosome was cloned and sequenced using the polymerase chain reaction (PCR). Since Darzins had already published the sequence for wild-type *pilG* from PAO1 (8), it was a relatively simple matter to design primers for cloning the FA2 *pilG* gene.

The *P. aeruginosa* chromosome has a high G+C content and it was found that the chromosomal DNA was difficult to fully denature so that the desired sequence was available as a template for a polymerization reaction. The template DNA was found to be more efficiently denatured and product more readily obtained during PCR when the chromosome was first digested with a restriction enzyme that did not cut within the *pilG* fragment. Fig. 4.15 shows the 0.7 kb PCR products obtained when PAO4141 and FA2 chromosomal DNA digested with Pst I were used in the PCR reactions. Optimization of reaction conditions determined that the best desired/non-specific product ratio was obtained with chromosomal DNA digested with Pst I when 100-200 ng of PAO4141 DNA and 250-400 ng of FA2 DNA were used for 25 PCR amplification cycles.

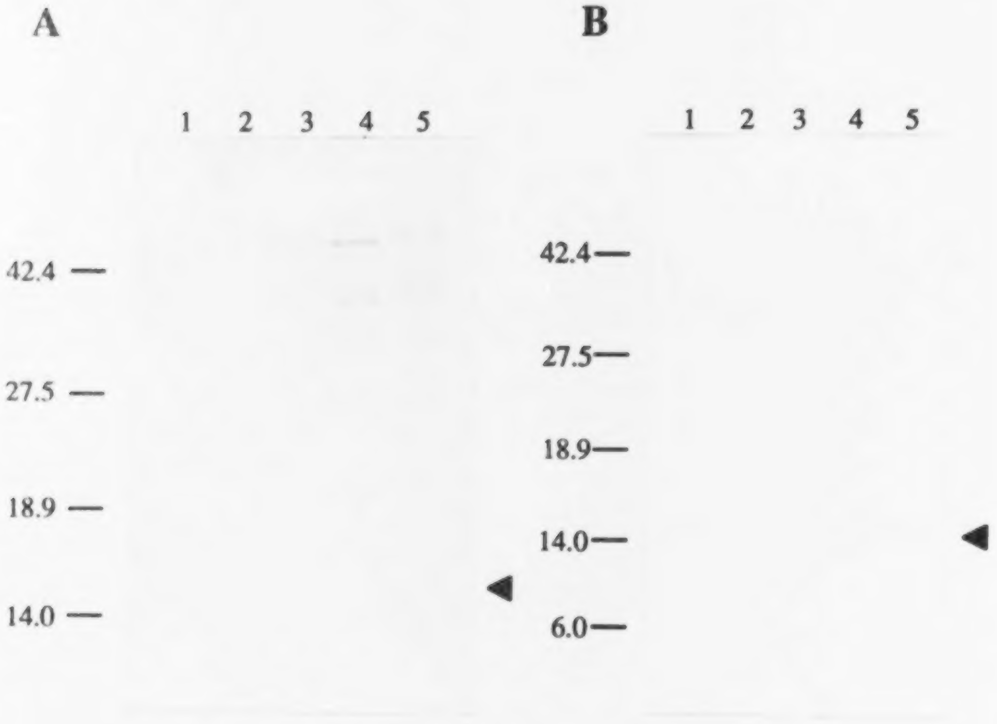


Fig. 4.14. Detection of pilin in filtrates from *P. aeruginosa* strains. Filtrate precipitate preparations (50 μg total protein) from PAO1, PAO4141, FA2, FA3, and the hyperpiliated mutant DB2 were separated on a 15% (w/v) SDS-PAG and stained with Coomassie Blue (**A**). The proteins from duplicate wells were electrophoretically transferred to a nitrocellulose membrane and pilin bound to the nitrocellulose was detected with pili-specific preadsorbed whole serum against PAO1 (**B**). Lanes: (1), PAO1 filtrate; (2), PAO4141 filtrate; (3), FA2 filtrate; (4), FA3 filtrate; (5), DB2 filtrate. Molecular weight standards (kDa) are shown on the left. Arrowheads indicate the positions of pilin bands.

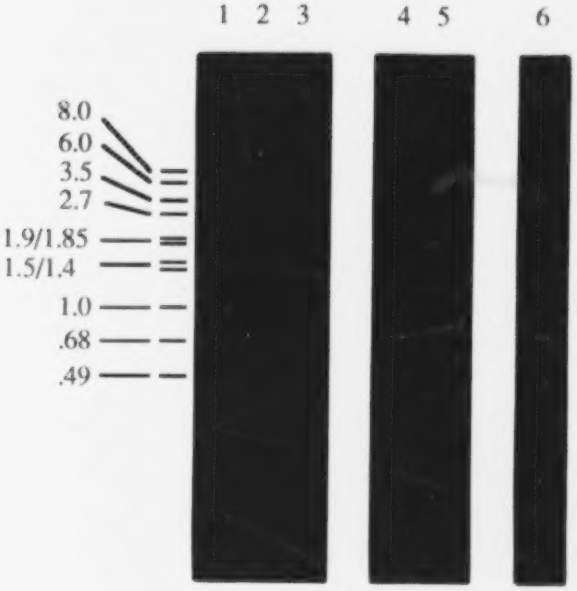


Fig. 4.15. PCR products from P. aeruginosa chromosomal template DNA digested with Pst I. PAO4141 and FA2 chromosomal DNA was digested with Pst I for use as templates for the amplification of the pilG gene. Bands were obtained that were the same sizes (0.7 kb) as expected for the Eco RI-Hind III fragment containing pilG. Lanes: (1), 500 ng PAO4141 template; (2), 100 ng PAO 4141 template; (3), 10 ng PAO 4141 template; (4), 750 ng FA2 template; (5), 500 ng FA2 template; (6), 250 ng FA2 template.

The 0.7 kb products from these reactions were purified and cloned into the Eco RI and Bam HI sites in the vector pBluescript SK+. Six PAO4141 clones and six FA2 clones were characterized by digesting the plasmid DNA with Sal I which cut once in the *pilG* gene and once in the vector and resulted in a 3.4 kb band and 0.3 kb band for clones containing a 0.7 kb *pilG* insert (Fig. 4.16). The inserts from the FA2 clones in lanes 2, 4, and 6 of the gel in Fig. 4.16 were sequenced as well as the inserts from the PAO4141 clone in lane 7 and another PAO4141 clone not shown. One typical *pilG* clone of each of FA2 and PAO1 was chosen and named pBPG-01 and pBPG-21, respectively. The PAO4141 clones showed no deviation from the *pilG* sequence published for PAO1 (8), confirming that PAO4141 has a *pilG* sequence identical to PAO1. The FA2 clones all had a single base change at position +268 as shown in Fig. 4.17. The transition of an adenine (A) to guanine (G) in this position resulted in an amino acid change of lysine to glutamate. If PilG is indeed a member of a signal transduction cascade as suggested by Darzins (8), the change of a basic to an acidic amino acid must alter the PilG protein enough to affect the signalling pathway necessary for normal expression of pili in *P. aeruginosa*. This change does not lead to an obvious difference in the overall character of the protein as seen in the hydropathy plots in Fig. 4.18, but it may affect the conformation of an active site in the mature, folded protein, giving rise to the phenotypic differences seen between the FA2 *pilG* mutant and its wild-type parent, PAO1.

## D. Conclusions

### I. Further Characterization of the *pilG* Mutants.

The results of experiments to further characterize two different types of *pilG* mutants suggested that the PilG protein may still retain some level of function in the FA2 mutant but not in FA3. FA2 may, as theorized by Darzins (8), produce a "pre-pilus" structure consisting of an exposed pilus tip at the surface of the bacterial cell. This is consistent with the phage sensitivity data for FA2 which, for the four phages tested in this study, was identical to the phage sensitivity pattern of its wild-type parent, PAO1. A very small percentage of cells were also observed to have a single pilus which could indicate an ability to synthesize pili far less efficiently than bacteria with a wild-type PilG protein. It is also possible that these piliated cells could be spontaneous *pilG* revertants and this was not ruled out by the investigation. Experiments designed to test the abilities of FA2 and PAO1 PilG proteins to be phosphorylated might provide more information about the function of PilG. The FA3 *pilG* insertion mutant was resistant to all phages which led to the conclusion that FA3 was probably non-piliated. These results seemed to indicate that the *pilG* mutation

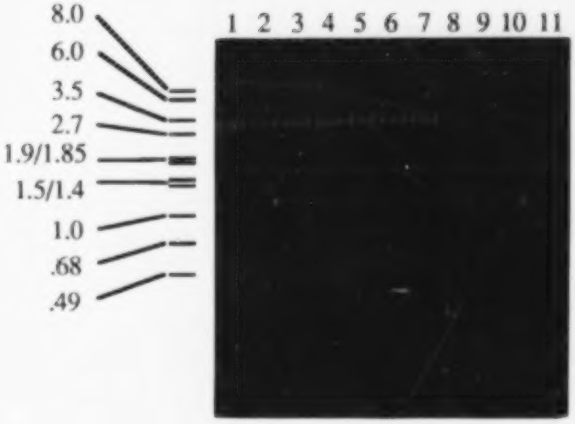


Fig. 4.16. Sal I digestions of potential pilG-containing clones. Plasmid DNA of potential pilG clones was digested with Sal I, electrophoresed on a 1.5% (w/v) agarose gel, and stained with ethidium bromide. All pilG -containing clones produced 0.3 kb and 3.4 kb bands. Lanes: (1), clone L1-01; (2), clone L1-15; (3), clone L1-22; (4), clone L1-24; (5), clone L1-31; (6), clone L3-01; (7), clone L3-03; (8), clone L3-04; (9), clone L3-05; (10), clone L3-06; (11), clone L3-07. Molecular weight standard (kb) shown at left.

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Eco RI
gaa ttc ctc cga aag ggg gtg ggt tgt ccg gtc gcg gcg ggg agg ccg cgt ggc aag cgg
1

gtt tga cgc tca ggt tgg cgc ccc gtt tgc gct tgg tca agg aaa aag ccg gcc tgt tcc
61

ggg cat tat gga tag agt tgc gtc gag tgt gct aaa aag gct cgc gat tgg gtc gca gcc
121

cgt cgg tga cag ggc gtt agc gca ctg aag aaa aca cag aag aat gat tca ccg agt cga
181

    SD          pilG                    7
tat agg gcg aac atg gaa cag caa tcc gac ggt ttg aaa gtg atg gtg atc gac gat tcg
                M   E   Q   Q   S   D   G   L   K   V   M   V   I   D   D   S
241

17                                      27
aaa acg att cgt cgc acc gcc gaa aca ctg ctg aaa aag gtc ggt tgc gac gtg atc acg
K   T   I   R   R   T   A   E   T   L   L   K   K   V   G   C   D   V   I   T
301

37                                      47
gca atc gac ggc ttc gat gcc ctg gcg aag atc gcu gat acc cat ccg aac atc att ttc
A   I   D   G   F   D   A   L   A   K   I   A   D   T   H   P   N   I   I   F
361

57                                      67
gtc gac atc atg atg ccg cgc ctg gat ggc tat cag acc tgc gcc ctg atc aag aac aac
V   D   I   M   M   P   R   L   D   G   Y   Q   T   C   A   L   I   K   N   N
421

77                                      87          g
agc gcg ttc aag tcc acc ccg gtg atc atg ctg tcc tcc aag gac ggc ctg ttc gac aag
S   A   F   K   S   T   P   V   I   M   L   S   S   K   D   G   L   F   D   K
481                                                 E

97                                      107
gcc aag ggg cgc atc gtc ggc tcc gac cag tac ctc acc aag ccg ttc agc aag gaa gag
A   K   G   R   I   V   G   S   D   Q   Y   L   T   K   P   F   S   K   E   E
541

117                                     127
ctg ctg ggc gcg atc aag gca cac gta ccc agc ttc acc ccg gtg gac gcc gtt tcc tga
L   L   G   A   I   K   A   H   V   P   S   F   T   P   V   D   A   V   S   *
601

                                                    Bam HI
tat ccg gcc gca tgg ccg ttg atg ctt att tcg tga tgg gga tcc
661
```

Fig. 4.17. Nucleotide sequence of the cloned *P. aeruginosa pilG* fragment. Nucleotide numbering begins at the Eco RI site and appears below the sequence. Amino acid numbering begins with the first residue (below the gene designation) and appears over the sequence. The putative ribosome binding site identified by Darzins is overlined and indicated by SD (8). The double-overlined sequence is the site of the mγδ-1 insertion in the FA3 *pilG* gene. The boxed residue indicates the single base change (adenine to guanine) in the FA2 *pilG* gene that resulted in a lysine to glutamate amino acid change. The mutant base and amino acid appear above and below the sequence, respectively.

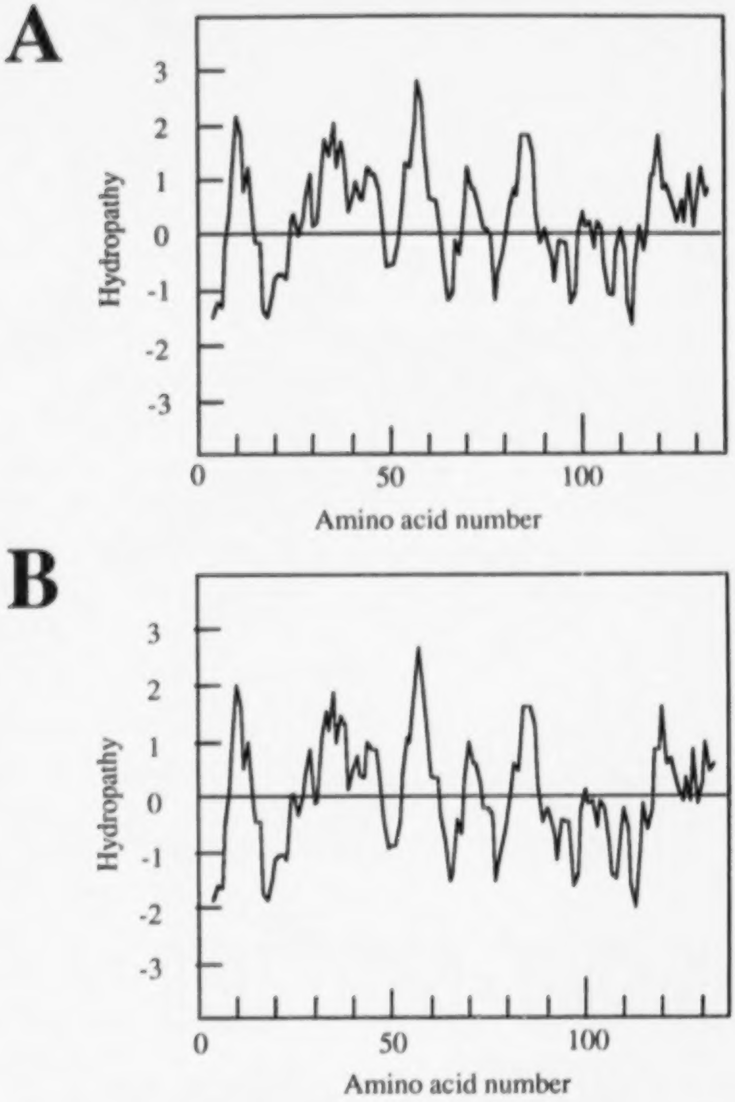

Fig. 4.18. Hydropathy profiles of wild-type (**A**) and mutant (**B**) PilG proteins. Hydropathy plots were generated using the Kyte-Doolittle scale and a window of 7 amino acids (21). Local minima indicate hydrophilic regions more likely to be surface exposed and maxima indicate more hydrophobic regions with a higher probability of being buried inside the protein structure.

prevented elongation of the pilus, as Darzins suggested, rather than preventing the assembly of proteins involved in the formation of a pilus base in the membrane and that the mutant PilG protein may still retain a low level of activity in FA2.

### II. Effects of *pilG* Mutations on *P. aeruginosa* Adherence and Virulence.

A comparison of virulence characteristics of *P. aeruginosa* PAO1 and FA2 revealed more similarities than differences between these strains. Both had almost 50 receptors per A549 cell despite the fact that FA2 was not observed to produce normal pili. The $LD_{50}$ obtained for FA2 in the A.BY/SnJ mouse model was only 1.2X greater than the PAO1 $LD_{50}$ and their $K_D$'s were also similar (see Table 4.3). By way of comparison, PAO-MP, a mutant whose pili did not express the disulfide loop adherence domain (Chapter 3.C.IV), had at least 6X fewer receptors per A549 cell than PAO1 or FA2. It was unlikely that the A549 human lung pneumocyte adherence assay selected for spontaneous *pilG* revertants since no significant bacterial growth would have occurred over the course of the assay. This would seem to indicate that the FA2 mutant retained most of its capacity for adherence and virulence in the mouse model even though it was unable to produce cells with normal pili, indicating that Darzins' suggestion that only a pilus tip is exposed at the FA2 cell surface is valid (8).

In comparing the virulence characteristics of *P. aeruginosa* PAO4141 and the *pilG* insertion mutant FA3, the lack of PilG in FA3 resulted in a lower number of receptors per A549 cell in the adherence assay as well as a 6X higher $K_D$. An $LD_{50}$ determination was not carried out with FA3 since the value obtained for its parent strain, PAO4141, was very close to the limit of detection in the A.BY/SnJ mouse model. The $K_D$ for FA3 would be expected to be at least as high or higher than the $K_D$ for PAO4141 since FA3 had even less ability to adhere than PAO4141. To make the comparison of results easier, it would be beneficial to construct a *pilG* gene replacement mutant in the PAO1 background. PAO4141 was observed to be far less virulent and far less able to bind in the A549 adherence assay than PAO1, making a direct comparison of the FA2 and FA3 mutants difficult. It does appear, however, that the disruption of the *pilG* gene had a more profound affect on adherence and probably virulence than the *pilG* point mutation.

### III. Effects of *pilG* Mutations on Pilus Assembly.

As mentioned previously, Darzins suggested that mutants in *pilG* are unable to elongate the pilus structure (8). Experiments carried out in this study support this hypothesis by providing more information about the cellular location of pilin protein in the mutants. Electron microscopy and twitching motility assays confirmed the lack of piliation in the mutants, while the phage sensitivity studies noted in section 5.D.I provided insight into the fact that there may still be a pilus base structure formed in the *pilG* mutants. The FA2 *pilG* point mutant possibly forms a "pre-pilus" structure that protrudes slightly from the cells and allows wild-type phage sensitivity, whereas the FA3 *pilG* insertion mutant appears to be unable to assemble pili at all. This could be due to a low level of function from the FA2 *pilG* point mutant compared to an absence of PilG in the FA3 disruption mutant. Some of the pilin processing machinery of the cells is still functioning since pilin was found to be located in the membranes of wild-type and mutant bacteria. It is possible, in the case of FA2, that the mutant PilG can allow polymerization of the pilin subunits into a structurally very weak pilus. This weak pilus could be mechanically unstable and extremely prone to breakage at or close to the surface of the bacterial cell such that some pilus-specific phages would have greatly reduced infectivity. This seems unlikely, however, since this would result in a significant amount of free pilus fragments in TEM preparations and this was not observed. Also, if this were the case, there would be more pilin detected in FA2 lanes than FA3 lanes in the shear fluids and culture filtrates Western immunoblots and again, this was not observed. Another possibility for FA2 was suggested by the observation of one or two bacteria with a single pilus in TEM studies: the mutant PilG protein may be able to provide a low level of activity; enough to assemble pili only very inefficiently. If the PilG protein functions in a manner similar to CheY, studies on the ability of the mutant and wild-type PilG proteins to be phosphorylated would provide more information about the role played by PilG in pilus assembly.

### IV. Comparison of PilG and CheY Structure.

The apparent similarities between CheY from *S. typhimurium* and the *P. aeruginosa* PilG protein have been previously mentioned in section 5.A.III. The point mutation in the *pilG* gene of FA2 resulted in a single amino acid change: Lys-90 to Glu. The PilG Lys-90 residue is conserved as Lys-92 in CheY in the alignment suggested by Darzins, which is shown in Fig. 4.1 (8). Observations made by Sanders et al (26) would seem to indicate that there are many amino acid residues in the area of the CheY phosphorylation site that play an important role in the conformation of that site. Another residue on the same face of the molecule as the phosphorylation active site is Lys-92, which occurs in the loop between the β4 strand and the αD helix. It is possible that this residue, or its corresponding residue in PilG, Lys-90, may play a minor structural role such as stabilizing the conformation of the phosphorylation site or interacting with CheA to facilitate phosphotransfer. This would account for the fact that the Lys-90 to Glu mutation in PilG does not seem to destabilize the protein but it does interfere with its ability to carry out its function in pilus assembly in FA2. One possibility is that if the Asp-58 residue of PilG can be phosphorylated like its Asp-57 counterpart in CheY, then Asp-58 is not phosphorylated with the same efficiency in the FA2 PilG point mutant as in the wild-type PilG protein. This may lead to a low level or complete absence of phosphorylated PilG in the cell. Since phosphorylated PilG could be part of the signal for assembly of pilin subunits into pili, the absence of this signal might lead to the inability to assemble the pili.

Assuming that the PilG protein has a similar structure to the CheY protein, the most likely explanation of the phenotype observed in the FA2 mutant is that the Lys-90 to Glu mutation in the FA2 PilG protein destabilizes the acidic pocket where phosphorylation of Asp-58 may take place, possibly by interacting with a residue important for the acidic character of the pocket, and resulting in a small change in conformation of the site or simply a change in the acidic character of the pocket. This small change to the PilG protein may result in a PilG that is inefficiently phosphorylated compared to wild-type PilG, resulting in a very low level of phospho~PilG and the production of Darzins' "pre-pilus" but few or no elongated pili. In contrast, the FA3 mutant does not produce any functional PilG protein, probably resulting in only a collection of processed pilin subunits in the membrane. This would account for the differences in phage sensitivity and adherence to A549 cells between FA2 and FA3. Recognizing that the FA2 and FA3 *pilG* mutants were in different background strains, it was difficult to make direct comparisons of their abilities to adhere to A549 cells, although it can be said that FA2's adherence relative to its parent PAO1 was better than FA3's adherence relative to PAO4141. Since the PilG protein itself is not yet well characterized, it would be interesting to determine the abilities of the mutant and wild-type PilG proteins to chelate a divalent metal ion in the acidic pocket as well as the abilities of the two proteins to be phosphorylated. This information would result in a much clearer picture of the actual nature of the change induced by the Lys-90 to Glu mutation in the FA2 PilG protein.

One other question remains as to the way in which PilG might control pilus assembly. Several gene products have been identified in *P. aeruginosa* that share sequence similarities with other signal transduction and chemotaxis proteins (9, 10). These include PilH (significant amino acid identity with the enteric CheY), PilI (significant amino acid identity with FrzA, a CheW homologue of *Myxococcus xanthus* ), PilJ (significant amino acid identity with the enteric serine receptor, Tsr), and PilK (significant amino acid homology to the enteric CheR). It is possible that these proteins could interact to regulate *P. aeruginosa* pilus assembly similar to the manner in which the enteric chemotaxis proteins interact to control flagellar rotation. PilJ could function as a membrane receptor for a signal to assemble pili as the homology between this protein and Tsr was found to be greatest within the C-terminal cytoplasmic (effector) domain, and sparse within the periplasmic (ligand-binding) domain. An insertion mutant in the *pilJ* gene resulted in the absence of pili, a loss of twitching motility, and an alteration in pilus-specific phage sensitivity (9). An insertion mutant in *pilI* resulted in an identical phenotype. The PilI protein appears to be a CheW homologue and could interact with the putative PilJ receptor upon receptor stimulation. This would be consistent with the identical phenotypes of the *pilI* and *pilJ* insertion mutants (9).

A *pilK* deletion mutant was found to be indistinguishable from wild-type but the deduced amino acid sequence of the PilK protein contained significant homology to CheR of *E. coli*. Several possible explanations for the lack of a PilK mutant phenotype were advanced although no evidence was presented for or against these possibilities (10). Like PilG, PilH was shown to have significant amino acid sequence homology with CheY, although, unlike the *pilG* mutants, the *pilH* insertion mutant was piliated but exhibited an altered form of twitching motility resulting in large swirls (9). A possible explanation for the existence of PilG and PilH is that each protein may interact with the pilus assembly machinery in a different way. PilG (phosphorylated) may be a signal for continued pilus elongation while PilH may control the "direction" of twitching motility. This is consistent with the observation that the absence of PilH resulted in an uncharacteristic swirling at the edge of zones of twitching motility but the absence of PilG resulted in an absence of pili and an altered pilus-specific phage sensitivity pattern. One thing that is clear is that many more genes affecting pilus biogenesis await discovery and much more work remains to be done to define the roles of individual gene products in this process.

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# Chapter 5

# Conclusion

# A. Discussion

## I. Introduction.

To begin to study pilus-mediated adherence of *Pseudomonas aeruginosa*, a method of bacterial enumeration using bacterial bioluminescence was investigated. The outcome of this line of experiments eventually led to the development of a standardized bacterial adherence assay involving the attachment of bacteria to monolayers of lung epithelial cells. This adherence assay allowed useful comparison of different strains of *P. aeruginosa* as well as contributing to the characterization of various adherence and pilus-assembly mutants by providing information about the binding characteristics of these strains. The investigation of adherence in pilus-assembly mutants led to efforts to further characterize a unique pilus assembly mutant that appeared to be unable to synthesize pili but retained many characteristics of piliated organisms. The discussion which follows will briefly summarize the main results and conclusions of this thesis, compare them to the work of other researchers in this field, and suggest possible directions for future experiments in these areas.

## II. Bacterial Enumeration Using Bioluminescence.

Bioluminescence from *P. aeruginosa* expressing *V. fischeri lux* genes was investigated as a method for enumerating the bacteria in aqueous suspensions (Chapter 2). It was hoped that this method would ultimately be applied to the enumeration of adherent bacteria in eukaryotic cell-binding assays. A *lux* gene cassette including five genes necessary for light production under control of the pBR322 tetracycline promoter for constitutive expression (13) had been cloned into a low-copy-number plasmid and transformed into several *P. aeruginosa* strains. When light production from these strains was assessed, it was found that there was a linear relationship between light output and the concentration of bacteria in a liquid suspension. This was in agreement with the results of Rattray *et al* (1990) who found that for bacteria expressing *V. fischeri lux* genes constitutively, light output per cell was independent of cell concentration and could be reliably used to assess cell concentration of bacteria recovered from an environmental soil sample (12). Light ouput was found to be further stabilized (at constant temperature) by including a small percentage of growth media in the buffer used for light measurements. Presumably this allowed the bacteria to continue to produce gene products necessary for light production but was not enough to support growth. The benefits of using this system for enumeration included the ease of manipulation for obtaining results as well as the fact that only viable bacteria would be counted by this method since only living bacteria were able to produce light.

Reproducible light output from *P. aeruginosa* expressing *lux* genes was also found to be dependent on temperature as the level of light output was observed to decrease with increasing temperature above room temperature. Later work by Meighan (1991) indicated that the *V. fischeri* luciferase was unstable at 37°C and this was likely the cause of the unstable luminescence (11). This also resulted in unstable light output when bacteria were subject to any kind of temperature change. This instability and an inability to make use of the *V. fischeri* reporter system at human physiological temperature forced the choice of another bacterial enumeration method for use in adherence assays. As mentioned in Chapter 2, with the information now available, it would probably be possible to make a luminescence enumeration system work. *Xenorhabdus luminescens lux* genes would be the best candidate since the thermal stability of its luciferase is much higher than that of *V. fischeri*'s luciferase (11).

### III. Pilus-Mediated Adherence of *Pseudomonas aeruginosa.*

The development of an adherence assay was undertaken to better characterize the attachment of *P. aeruginosa* to an epithelial cell monolayer and to allow comparisons between different strains or adherence mutants. Two existing adherence assays for *P. aeruginosa* to epithelial cells had limitations it was important to address: viability of the epithelial cells and viability of the bacteria during the course of the binding assay. Irvin *et al* (1990) designed an adherence assay that measured the association of *P. aeruginosa* PAK and PAO with human buccal epithelial cells, of which only 5% were viable at the time of the assay (8). The limitation involved was that with epithelial cells in different states of viability, there was likely a varied cell surface presented to the bacteria and some structures may have been available as receptors that might not have been available on the surface of a living, functioning cell. Chi *et al* (1991) designed an adherence assay using human lung pneumocyte monolayers, but the bacterial enumeration method involved staining and counting the bacteria in the microscope, an arduous task that did not differentiate between viable and non-viable bacteria (1). The development of an adherence assay using human lung pneumocyte monolayers combined with a plate count method for bacterial enumeration provided the desired parameters for investigating *P. aeruginosa* attachment (Chapter 3).

As the first step in bacterial pathogenesis, bacterial attachment to host surfaces is an interesting and important interaction to characterize. Pili had already been shown to be important for adherence of non-mucoid *P. aeruginosa* to human buccal and tracheal epithelial cells (4, 17). The effect of pilin subunit structure on adherence had also begun to be investigated, especially the role of the C-terminal disulfide loop in adherence (9, 10, 14). It had not, however, been directly demonstrated that the disulfide loop was necessary for adherence to epithelial cells. A mutant strain of *P. aeruginosa* PAO1 was constructed that produced pilin without the C-terminal disulfide loop. This mutant was able to synthesize pili and was sensitive to pilus-specific bacteriophages but it was not able to adhere well in the human lung pneumocyte binding assay, nor was it virulent in a mouse model. This study provided the first direct evidence that the disulfide loop of the pilin protein was not required for proper assembly of a pilus but it was required for adherence to human lung pneumocyte monolayers and for virulence (5, Chapter 3). In the case of cystic fibrosis lung infection, it has been observed that the colonizing strains of *P. aeruginosa* are non-mucoid and undergo a subsequent conversion to mucoidy (6, 7), indicating that pili are probably the main adherence factor in establishing infection. This underlines the importance of continued investigation of therapies aimed at preventing pilus-mediated attachment as the first step of that infection process.

### IV. Pilus Assembly in *Pseudomonas aeruginosa.*

To date, 22 genes involved in pilus expression have been identified. Some have been well characterized, but most await further experimental evidence of their roles in pilus biogenesis. The *pilG* gene was identified by Darzins (1993) when he isolated a spontaneous *P. aeruginosa* PAO1 mutant, apparently unable to assemble pili and incapable of twitching motility but sensitive to some pilus-specific bacteriophages. Darzins suggested that this might be due to the expression of only the tips of pili at the bacterial cell surface rather than fully assembled and functional pili. Comparison of the deduced amino acid sequence of wild-type PilG to sequences in the protein databases also led Darzins to find that PilG shared significant similarity with a subclass of response regulator (RR) proteins that consisted of only the single N-terminal RR domain which had kinase activity but no DNA-binding activity. Specifically, PilG was found to have 32% amino acid identity with the enteric chemotaxis regulator, CheY. Another *P. aeruginosa* mutant that did not produce any PilG was phenotypically identical to other non-piliated mutants that did not assemble pili, were incapable of twitching motility, and broadly resistant to pilus-specific bacteriophages.

Experiments were undertaken to further characterize the effect of the *pilG* mutations on attachment and virulence of *P. aeruginosa*. Transmission electron microscopy (TEM) studies indicated that both mutants appeared, for the most part, to be non-piliated except that in the case of the spontaneous *pilG* mutant a cell with a single pilus was occasionally observed (Chapter 4). This result suggested that a low level of PilG function in the mutant might be enough to allow occasional assembly of a pilus rather than the surface-exposed pilus tip phenotype suggested by Darzins. More extensive TEM studies showing bacteriophage attachment to either cell surfaces or infrequent pili will be required to distinguish between these possibilities. The effect of the spontaneous *pilG* mutation on bacterial attachment and virulence was minimal as the mutant was able to adhere to human lung pneumocytes at a wild-type level and its $LD_{50}$ was only 1.4X greater than wild-type. Evidently some form of pilus is expressed in this mutant since virulence and attachment were relatively unaffected in spite of the lack of obvious pili. Comparisons with the *pilG* disruption mutant were difficult since this mutant was constructed in a different background strain with different binding and virulence characteristics than PAO1. It would be beneficial to construct this mutant in the PAO1 background to allow a direct comparison of the effect of disrupting the *pilG* gene with the spontaneous mutant.

After sequencing the *pilG* gene from the spontaneous mutant, extensive comparison of PilG and CheY amino acid sequences was undertaken. This PilG mutant was a point mutant with a single amino acid change in a position that would be close to the active site of the CheY protein. CheY is phosphorylated at its Asp-57 residue which is conserved in PilG as Asp-58 (2). Although phosphorylation of PilG has not been demonstrated, it is attractive to speculate that PilG could be phosphorylated and may perform a regulatory function in the assembly of pili in a manner similar to the function of CheY in controlling the direction of flagellar rotation in enteric chemotaxis (15, 16). It is possible that the Lys-90 to Glu mutation in PilG might destabilize a phosphorylation site such that PilG would only be phosphorylated and become active at a very low level in the point mutant. If phosphorylated PilG is part of the signal pathway for assembly of pilin subunits into pili, the lack of this signal may lead to an inability to assemble pili. A low level of the signal may lead to occasional pilus assembly or assembly of only pilus tips at the cell surface (2, Chapter 4). The identification of other *P. aeruginosa* gene products that function in pilus biogenesis and share homology with other proteins of the enteric chemotaxis system suggests that a chemotaxis-like system may exist for pilus assembly (3). Isolation of the PilG protein and characterization of its ability to be phosphorylated will be necessary to further characterize the role of this protein as a putative response regulator in *P. aeruginosa* pilus assembly.

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